SEXUAL PATTERNS OF MONOOXYGENASE FUNCTION IN THE LIVER OF MARINE TELEOSTS AND THE REGULATION OF ACTIVITY BY ESTRADIOL

by

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ABSTRACT

Sex differences in hepatic microsomal cytochrome P-450 and monooxygenase activities were investigated in the marine teleosts scup (Stenotomus chrysops) and winter flounder (Pseudopleuronectes americanus). Microsomal cytochrome P-450 content per unit protein was 3-6 times lower in gonadally mature females than males. Ethoxyresorufin O-deethylase (EROD) activity in microsomes from females of both species was less than or equal to activity in males, reflecting sexually differentiated levels of the responsible isozyme, P-450E. Estradiol (E_2) 2-hydroxylation, demonstrated here for the first time in teleost microsomes, was measured via ^3H_2O release from [2-^{3}H]E_2. Microsomal E_2 2-hydroxylase activity in scup was P-450-mediated, although not by P-450E, and was 2-fold lower per unit protein in females than in males. Testosterone 6β-hydroxylase and aminopyrine N-demethylase (APDM) activities in scup were not sexually differentiated. In winter flounder microsomes, E_2 2-hydroxylase, testosterone 6β-hydroxylase, and APDM activities were all sexually differentiated. These three activities were decreased 2-3 fold per unit protein and increased 2-4 fold per unit P-450 in gonadally mature female winter flounder.

Levels of microsomal P-450E and P-450A were quantified by immunoblot. Specific P-450E content was lower in females than in males of both species, but P-450E per nmol P-450 was sexually differentiated only in winter flounder, where it was decreased in females. P-450A per unit protein was not sexually differentiated in either species, and in scup was not differentiated per nmol P-450. However, in winter flounder P-450A per nmol P-450 was five times greater in females than in males. Previously, reconstituted scup P-450A catalyzed both testosterone 6β-hydroxylase and E_2 2-hydroxylase activities (Klotz et al., Arch. Biochem. Biophys., 249 (1986): 326). P-450A levels were positively correlated to some extent with these two activities and APDM, suggesting co-regulation with or catalysis by P-450A.

E_2 injections suppressed microsomal monooxygenase activities and P-450E levels per unit protein in gonadally regressed winter flounder. Qualitatively, this change was like the decreased activities in female winter flounder. Other characteristics of the female-type pattern of monooxygenases were not reproduced by E_2 treatment. This suggests that E_2 could regulate monooxygenase activities in gonadally mature female winter flounder, but indicates that additional factors are also involved. It is speculated that testosterone or 17α,20β-dihydroxyprogesterone, which are elevated in plasma of spawning female teleosts, may also be regulatory. In rats and mice, sex differences in cytochrome P-450 are imparted by pituitary growth hormone and by the male sex steroid testosterone. In teleosts, sex differences in hepatic monooxygenases could be effected by means other than those known to function in mammals.

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I'm not the first to notice this, but thesis research is not something you can do solo. Bruce Woodin, Pam Kloepper-Sams, Adria Elskus, Jay Gooch, Alan Klotz, Frances Teng, Yvette Weider, and Kate Goddard-Doms all had a hand in fish collection and sample analysis, and at times allowed me to pick their brains and use their own samples. The above mentioned people, as well as The Knights of the Round Table at Camelot (especially Phil), the denizens of Carey Manor, and the nicknamed derelicts at MIT are also acknowledged for providing entertainment through the Biology softball team, the Leaping Lizards volleyball team, countless camping and fishing trips, and the knitting group. I want to acknowledge my family for their unflagging support, and finally, Jeff, for providing encouragement, insight, and humor every step of the way. If we weren't already married, I would have proposed by now. After all, it is a leap year.

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There is something fascinating about science. One gets such wholesale returns of conjecture out of such a trifling investment of fact.

Mark Twain

Life on the Mississippi

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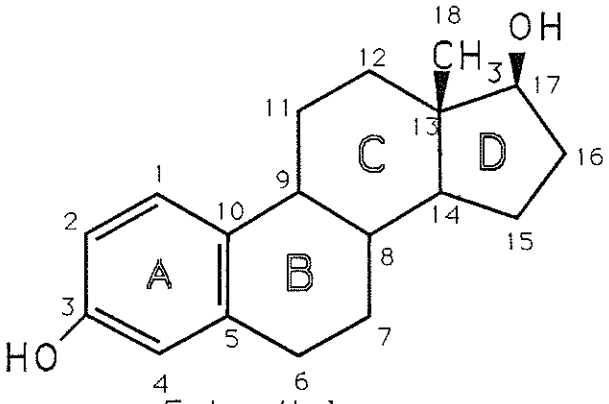
ABBREVIATIONS

AHH, aryl hydrocarbon (benzo[α]pyrene) hydroxylase
APDM, aminopyrine N-demethylase
BNF, β-naphthoflavone
BP, benzo[α]pyrene
DMSO, dimethylsulfoxide
E_2, estradiol (estra-1,3,5(10)-triene-3,17β-diol)
2-OH-E_2, 2-hydroxyestradiol
EM, ethylmorphine
ECOD, 7-ethoxycoumarin O-deethylase
EROD, 7-ethoxyresorufin O-deethylase
GH, growth hormone
  hGH, human growth hormone
  rGH, rat growth hormone
GSI, gonadosomatic index
GtH, gonadotropic hormone
HPLC, high performance liquid chromatography
HSI, hepatosomatic index
HSOR, hydroxysteroid oxidoreductase
i.p., intraperitoneal
kD, kilodalton
MAb, monoclonal antibody
MC, 3-methylcholanthrene
MW or M_r, molecular weight
NADH, reduced nicotinamide adenine dinucleotide
NADPH, reduced nicotinamide adenine dinucleotide phosphate
17,20β-P, 17α,20β-dihydroxyprogesterone (17α,20β-dihydroxy-4-pregnen-3-one)
PAH, polycyclic aromatic hydrocarbon
PB, phenobarbital
PCN, pregnenolone-16α-carbonitrile
PNAO, p-nitroanisole O-demethylase
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC, thin-layer chromatography

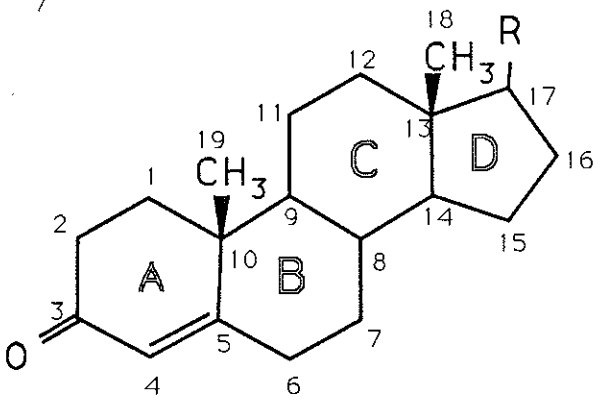
FISH NAMES OCCURRING IN THIS THESIS

Common name	Latin	Family
Bleak	Mirogrex terrae-sanctae	Cyprinidae
Stinging catfish	Heteropneustes fossilis	Heteropneustidae
Cod (Atlantic)	Gadus morhua	Gadidae
Cunner	Tautogolabrus adspersus	Labridae
Dogfish	Squalus acanthias	Squalidae
Silver eel	Anguilla anguilla	Anguillidae
Flounder	Platichthys flesus	Pleuronectidae
Winter flounder	Pseudopleuronectes americanus	Pleuronectidae
Goldfish	Carassius auratus	Cyprinidae
Red grouper	Epinephelus akaara	Serranidae
Hagfish	Myxine glutinosa	Myxinidae
Hagfish	Eptatretus burgeri	Eptatretidae
Jewel fish	Hemichromis bimaculatus	Cichlidae
Killifish	Fundulus heteroclitus	Cyprinodontidae
Sailfin molly	Poecilia latipinna	Poeciliidae
Plaice	Pleuronectes platessa	Pleuronectidae
Scup	Stenotomus chrysops	Sparidae
White sucker	Catostomus commersoni	Catostomidae
Tilapia	Oreochromis mossambicus	Cichlidae
Brook trout	Salvelinus fontinalis	Salmonidae
Rainbow trout	Salmo gairdneri	Salmonidae
Vendace	Coregonus albula	Coregonidae

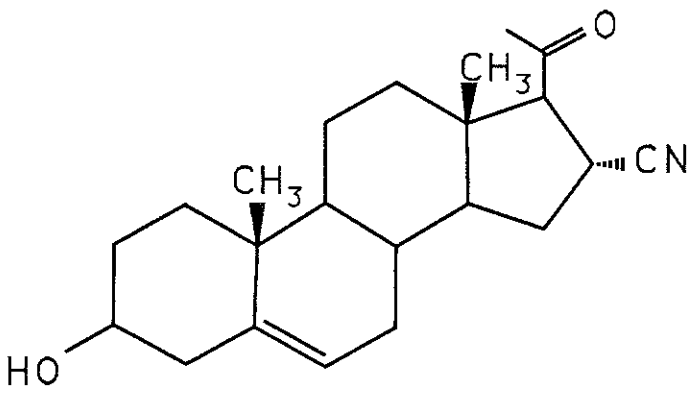
STRUCTURES OCCURRING IN THIS THESIS



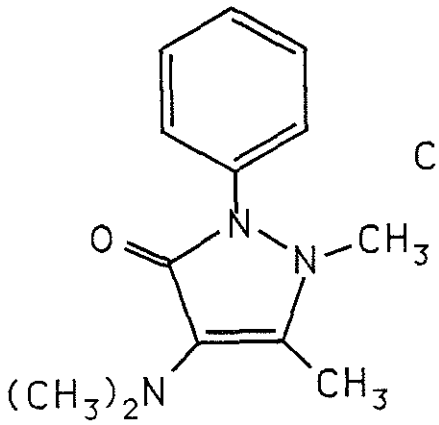
Estradiol



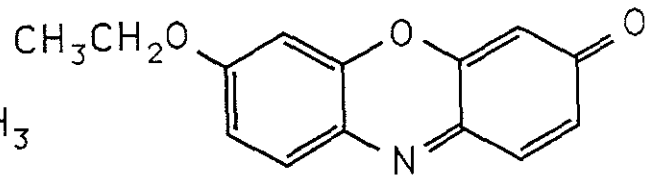
Testosterone, R = -OH
Androstenedione, R = =O
Progesterone, R = -COCH$_3$
(4-Pregnene-3,20-dione)



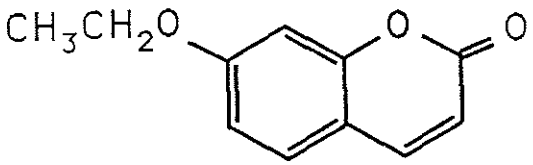
Pregnenolone 16[a]carbonitrile (PCN)



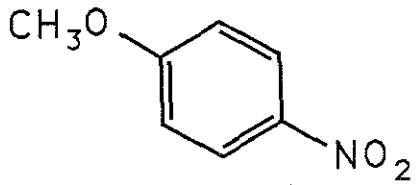
Aminopyrine



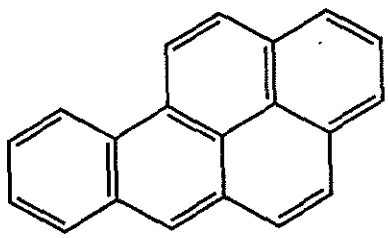
7-Ethoxyresorufin



7-Ethoxycoumarin



p-Nitroanisole



Benzo[a]pyrene

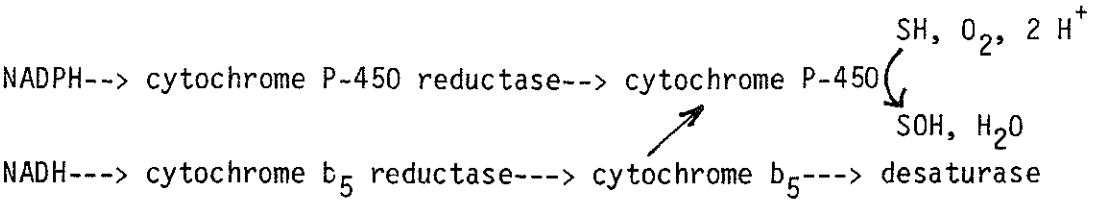
CHAPTER 1: INTRODUCTION

The Cytochrome P-450 Monooxygenase System

Sex-based differences in steroid and xenobiotic metabolism by the liver have been established in both mammals (rats, humans, and mice) and fish. Intensive studies on the nature of these differences have concentrated mainly on the rat, providing a model of the mammalian system. In this model the sex steroids, androgens and estrogens, do not impart the sex differences directly on the liver but appear to work through and be directed by the hypothalamo-pituitary axis (Gustafsson et al, 1980). Studies with teleosts have yielded a partial understanding of the nature of the control of sex-associated differences in metabolism in these animals as well; there appear to be substantial differences from the mammalian system. For example, while the patterns of metabolism are similar between mammals and teleosts, it appears in teleosts that in some instances estrogens may direct hepatic metabolism independently of the pituitary. The following research was directed toward furthering comprehension of sex differences in xenobiotic and steroid metabolism by teleosts, and regulation of metabolism by estrogens.

In many animal species, hepatic steroid and xenobiotic metabolism is accomplished to a large extent by the cytochrome P-450 containing oxidative enzymes (mixed function oxidases or monooxygenases) and the steroid Δ^4-hydrogenases. Cytochrome P-450 refers to a family of membrane-associated enzymes which in the liver are localized with the microsomal fraction of homogenates. In the CO-bound, reduced form cytochrome P-450 exhibits an absorption maximum near 450 nm (Omura and Sato, 1964). These enzymes are the terminal oxidases in numerous C-, N-, and S- hydroxylation and dealkylation reactions in which electrons from NADH or NADPH are channeled to molecular oxygen, activating and reducing it such that one oxygen atom is transferred to the substrate and the other is reduced to water. The membrane bound cytochrome P-450 receives reducing equivalents via the flavoprotein NADPH-cytochrome P-450 reductase, which may donate them to cytochrome c in an artificial system. Microsomal cytochrome P-450 may also receive electrons from NADH-cytochrome b_5 reductase via cytochrome b_5 (West and Lu, 1977); the in vivo significance is unclear. Cytochrome b_5 is also involved in the electron transfer for oxidative desaturation of fatty acids. A schematic illustration of

electron flow is shown below.



Whereas these electron transfer components are essential for cytochrome P-450 function, they do not participate directly in catalysis.

The microsomal monooxygenase system metabolizes a broad range of compounds, including steroids, fatty acids, and carcinogens (Conney, 1967). This unusual diversity was attributed to multiple forms of cytochrome P-450, a concept that has been confirmed by the purificatin of mammalian cytochrome P-450 isozymes distinguished by physicochemical characteristics including substrate specificity (Gustafsson and Ingelman-Sundberg, 1976; Thomas et al, 1976). Purified, reconstituted isozymes appear to display overlapping specificities for xenobiotic and steroid oxidation (for reviews see Lu and West, 1980; Schenkman et al, 1987; Waxman, 1988). The regulation of these multiple isozymes contributes to the complex nature of hepatic microsomal steroid and xenobiotic metabolism.

The rate at which a substrate is metabolized by microsomes is dependent on the levels of the cytochrome P-450 isozymes contibuting to the activity. Both exogenous and endogenous compounds can influence the quantity of specific cytochrome P-450 isozymes and subsequent catalysis. Drugs and environmental pollutants can stimulate the de novo synthesis, or induction, of one or more forms of cytochrome P-450. Induction of hepatic cytochrome P-450 has been documented in both mammals and fish (reviews: Lu and West, 1980; Stegeman, 1981). There are multiple inducers, and many can be categorized as either 3-methylcholanthrene (MC)-type or phenobarbital (PB)-type inducers. These two major classes can be distinguished on the basis of physicochemical and catalytic differences of the isozymes induced. Substances categorized as MC type inducers cause a shift in the reduced CO difference absorption spectra toward a maximum near 448 nm. This induction augments the metabolism of polyaromatic hydrocarbons (PAH)-type substrates and a variety of drugs in rats. PB induction elevates cytochrome P-450 levels but does not cause a

spectral shift. The N-demethylation of compounds such as ethylmorphine (EM) and aminopyrine is preferentially enhanced (Lu and West, 1980).

It is well-established that sex steroids regulate hepatic metabolism in the rat (Kato, 1974), attributable in part to sex-specific regulation of cytochrome P-450 isozymes (Kamataki et al, 1983; Waxman et al, 1985). The regulation of cytochrome P-450 by the sex steroids is effected exclusively through the pituitary (Colby, 1980; Gustafsson et al, 1980). In teleosts, steroid regulation of hepatic cytochrome P-450 and monooxygenase activity has also been described (Stegeman et al, 1982; Hansson, 1982), but the pituitary may not be an obligatory intermediary (Hansson and Gustafsson, 1981b). Futhermore, while androgens are the principal steroid regulators of cytochrome P-450 in mammals (Colby, 1980), estrogens seem to be the dominant effectors in teleosts (Stegeman et al, 1982). These distinctions were addressed in this study of sex differences in hepatic metabolism by the marine teleosts scup (Stenotomus chrysops) and winter flounder (Pseudopleuronectes americanus).

Review of Mammalian Literature

Much of the knowledge of the control of hepatic enzyme activities stems from research on mammalian species, of which the rat has been most thoroughly characterized. Many of the patterns of metabolism in mammals are apparent in fish, but the regulation of these patterns may be different between the two vertebrate groups. Therefore, it will be helpful to survey the literature of mammalian systems before proceeding with a review of teleost metabolism and its regulation.

Mammalian Sex Differences in Hepatic Monoxygenases

Xenobiotic metabolism: Sex differences in hepatic xenobiotic metabolism by monooxgenases in rats is well established, and are summarized on Table 1-1 (for reviews see Kato, 1974; Colby, 1980). The metabolism of the drugs aniline and zoxazolamine was similar between both sexes (Kato and Gillette, 1965). However, the metabolism of other drugs, such as hexobarbital (Quinn et al, 1958), ethylmorphine (EM) (Axelrod, 1956), and lidocaine (von Bahr et al, 1977), was consistently faster in liver slices and microsomes from male rats. Cytochrome P-450 content and NADPH-cytochrome c reductase activity were both higher in male rats than in females (Kato et al,

TABLE 1-1: SEX DIFFERENCES IN MAMMALIAN HEPATIC MICROSOMAL ACTIVITIES

Species	Microsomal Activity, unit per mg	Male vs. Female rates of activity
Rat		
	Aniline, zoxazolamine hydroxylation	male = female[1]
	Imipramine, hexobarbital, lidocaine hydroxylation	male > female[2]
	Ethylmorphine, aminopyrine demethylation	male > female[2]
	Steroid disulfate 15β-hydroxylation	male < female[3]
	Estradiol, 2-hydroxylation	male > female[4]
	Testosterone, 16α-hydroxylation	male > female[5]
	Testosterone, 5α-reductase	male < female[6]
Mouse		
	Ethylmorphine demethylation	male < female[7]
	Testosterone, 15α-hydroxylation	male < female[8]
Human		
	Estradiol, 2/16α-hydroxylation	male < female[9]
Tree shrew		
	Aniline, benzo[α]pyrene hydroxylation	male < female[10]

[1]Kato and Gillette, 1965
[2]Kato, 1974; von Bahr et al, 1977; Skett et al, 1980
[3]Gustafsson and Ingleman-Sundberg, 1974
[4]Conney et al, 1965
[5]Berg and Gustafsson, 1973
[6]Forchielli et al, 1958
[7]Castro and Gillette, 1967
[8]Ford et al, 1979
[9]Fishman et al, 1980
[10]Kramers et al, 1979a

1968; Schenkman et al, 1967) but these differences of only 30-40^{0}/o can not entirely account for the 2-4 fold increased rate of metabolism. Both the Michaelis constant, K_m, and the spectral dissociation constant, K_s, were higher for hexobarbital with microsomes from the female rat, but these constants were not significantly different between the sexes for aniline (Schenkman et al, 1967). This suggested that there was a greater affinity of the terminal oxidase in male microsomes for some xenobiotics, possibly from an enhancement of those forms of cytochrome P-450 with a high efficiency of substrate utilization.

Sex steroids were shown to mediate some pattern of xenobiotic metabolism. Castration reduced EM N-demethylase and hexobarbital hydroxylase activities in male rats, but testosterone treatment restored them (Quinn, et al, 1958; El Defrawy and Mannering, 1974). Testosterone administration to female rats increased the rate of microsomal drug metabolism to levels comparable with males (Axelrod, 1956; Quinn et al, 1958). Therefore, testosterone can induce and maintain a masculine pattern of metabolism. The influence of the female sex steroid estradiol (E_2) on metabolism is more complex. Castration of male rats reduced lidocaine N-deethylation, imipramine N-demethylation, and imipramine N-oxidation in male rats but ovariectomy of females did not affect these activities (Skett et al, 1980). Testosterone treatment increased aminopyrine N-demethylation (APDM) and hexobarbital hydroxylation in castrated male rats, but the increases were blocked by the simultaneous administration of E_2 or the synthetic estrogen diethylstilbesterol (Kato and Onada, 1970). The chronic administration of estrogens to male rats imparted partial feminization of activity (el Defrawy and Mannering, 1974). Hence, while estrogens are not crucial in maintaining a feminine pattern they can induce it from a masculine one.

EM metabolism in mice was also sex dependent but opposite from the rat pattern (Castro and Gillette, 1967). Hepatic microsomes from female mice of the CPB-SE strain had a higher specific content of cytochrome P-450 and higher EM demethylase activity than those from the male. Testosterone treatment reduced these factors in the female and increased the K_m for EM demethylase but was without effect in the male. Castration increased cytochrome P-450 levels and

demethylase activity and reduced the K_m in the male without changing them in the female (Noordhoek et al, 1978; van den Berg et al, 1977). All of this suggested that androgens repressed cytochrome P-450 levels and the hepatic metabolism of EM in the adult male CPB-SE mouse (van den Berg et al, 1978). In the CPB-V strain, cytochrome P-450 levels were somewhat higher in the microsomes from the male and were slightly increased in females by testosterone pretreatment. EM demethylase activity was not sexually differentiated and was unaffected by testosterone (van den Berg, 1977). The effects of testosterone, then, were less striking than in the SE mouse strain. However, the patterns of sex dependent EM metabolism in both mice and rats were dependent on androgens for manifestation.

Examples of sex differences in drug metabolism exist in other mammalian species, but are less pronounced than in the rat. As in the mouse, xenobiotic metabolism was more rapid in hepatic microsomes from female tree shrews than from males (Kramer et al, 1979a). Although EM demethylase activity was sexually differentiated in mice, the metabolism of other substrates such as aminopyrine, hexobarbital and hexobarbitone was not significantly different between males and females (Quinn et al, 1958; Kato et al, 1968). Furthermore, drug metabolism was not sexually differentiated in the dog, cat, guinea pig, rabbit or monkey (Quinn et al, 1958; Castro and Gillette, 1967; Conney, 1967). The species-associated manifestation of sex differences in xenobiotic oxidation is an important phenomenon in comparative metabolism.

Steroid metabolism: In rats, the metabolism of steroids, as well as drugs, displayed a masculine and feminine dichotomy. The in vitro reduction of the A ring of Δ^4-ketosteroids by liver homogenates from females was three to four times faster than the reduction by males (Forchielli et al, 1958). Castration of male rats increased this activity, and subsequent testosterone treatment decreased it to typical male levels (Yates et al, 1958). Steroid hydroxylase activities tended to have an opposite pattern---i.e., male hydroxylation rates were usually higher and castration resulted in a reduction of metabolism (Berg and Gustafsson, 1973; Einarsson et al, 1973b). One exception to this pattern was the 15β-hydroxylation of androstane-3α,17β-diol-3,17-disulfate, conducted at a much higher rate

in preparations from female rat livers than from males (Gustafsson and Ingleman-Sundberg, 1974).

E_2 and estrone were metabolized to polar products faster by hepatic microsomes from male rats than from females (Conney et al, 1965). This increased activity became apparent with sexual maturation and was testosterone dependent (Jellinck and Lucieer, 1965). The dominant hydroxylated products were the catechol estrogens 2-OH-E_2 and 4-OH-E_2, principally the former (Ball et al, 1978). Hepatic microsomes also hydroxylated E_2 at positions C-6α, -6β, -7α, and -16α, and oxidized it at C-17 to estrone (Ball et al, 1978; Gustafsson, 1978).

Pharmacologically, catechol estrogens may act as weak estrogens or anti-estrogens, inhibit drug and catecholamine metabolism, and bind with intracellular macromolecules in vitro and in vivo (Ball and Knuppen, 1979; Martucci and Fishman, 1979; Jellinck et al, 1981; Brueggemeir et al, 1984). The physiologial role of the catecholestrogens is unclear. 2-OH-E_2 is synthesized by microsomal cytochrome P-450 in many tissues, but activity is dominant in the liver, followed by the brain (Ball et al, 1978; Hoffman et al, 1980). Activity in male liver and brain microsomes was greater than in female tissues and was maintained at the higher levels by testosterone (Barbieri et al, 1978; Hoffman et al, 1980). Paradoxically, 17α-ethinylestradiol induced hepatic E_2 2-hydroxylase activity in ovariectomized female rats (Shiverick and Notelovitz, 1983). The authors suggested that this could represent multihormonal control of this activity in the female, i.e., indirect supppression through the pituitary and direct stimulation by admininstered ethynylestradiol, perhaps through an estrogen receptor. There is a possibility of a dual regulation of in vitro androstenedione metabolism in rainbow trout (Salmo gairdneri) (Hansson and Gustafsson, 1981b).

Sex differences in steroid metabolism have also been observed in humans (Pfaffenberger and Horning, 1977) but were not as marked as in rats. Oxidation of E_2 to estrone is the most rapid transformation of this steroid in both women and men, followed by 2-hydroxylation and 16α-hydroxylation. The rates of all of these reactions in premenopausal women were significantly greater than in men (Fishman et al, 1972, 1980). 15α-Hydroxylation of testosterone was greater in

hepatic microsomes from female mice than from males (Ford et al, 1979). This sex difference was attributed to cytochrome P-450_{15a}, a testosterone 15α-hydroxylase present at higher quantities in females. Sexual dimorphism in the levels of this protein and subsequent catalytic activity resulted from sexually differentiated levels of P-450_{15a} mRNA (Harada and Negishi, 1984; Burkhart et al, 1985).

Imprinting

In rats, the sex differences in the rates of steroid metabolism were not uniformly dependent on androgens for manifestation. The steroid metabolizing activities have been categorized into three general groups, based on patterns of response to androgens (Einarsson et al, 1973). In the first group the high levels of activity in the male were entirely dependent on the continuous presence of androgens. 2β- and 18-hydroxylation of 5α-androstane-3α,17β-diol and 6β-hydroxylation of androstenedione are examples of these activities. Castration of males reduced these enzyme activities to female levels, and testosterone treatment completely restores the male levels. The second group of activities reflects partial dependence on androgens. 2α- and 16α-hydroxylation of androstenedione exemplify this group. Castration of adult males resulted in only partial feminization of enzyme activities, but activities in adult males castrated as neonates were completely feminized. However, castrated neonates immediately treated with androgens develop only partial feminization as adults. Apparently, androgens in the neonate irreversibly "imprint" some tissue such that certain enzyme activities in the adult are partially masculinized even in the absence of testosterone. The third group of steroid hydroxylating activities, which includes 7α-hydroxylation of androstenedione and 16α-hydroxylation of 4-pregnene-3,20-dione, show little if any sexual differentiation and were almost or completely androgen independent.

Pituitary Control of Sex Differences

Sex differences in hepatic metabolism were at first attributed to the direct effects of the sex steroids on the liver. It was theorized that androgens bound to a hepatic receptor to cause de-repression of enzyme activity in the male (Kato and Onada, 1970; Kato, 1974). Neonatal imprinting was suggested to induce high concentrations of testosterone receptors in the adult liver for this purpose (Gustafsson

and Stenberg, 1974b). However, further research showed that steroids mediate hepatic monooxygenase activities via the pituitary gland. The sex differences in metabolism do not appear until 30 days of age and are not complete until 42 days of age, a period which approximates puberty and the maturation of the pituitary (Dohler and Wattke, 1974; Ojeda and McCann, 1974). Hypophysectomizing adult male and female rats abolished the sex differences in hepatic metabolism (Denef, 1974). Hypophysectomizing adult male rats has the same effect as castration with respect to lidocaine and imipramine metabolism (Skett et al, 1980). The mature pituitary, then, is requisite for sexual differentiation of metabolism.

The pituitary has been shown to be necessary to execute the opposite effects of administered estrogens and androgens on hepatic mixed function oxidases in males (Gustafsson and Stenberg, 1976; Kramer et al, 1979b). However, the pituitary alone is responsible for patterns of metabolism in adult female rats. Ovariectomy of females did not effect lidocaine and imipramine metabolism, but hypophysectomy caused a marked shift toward higher, male-type levels of activity (Skett et al, 1980). Hence, the pituitary seemed to be the major factor responsible for female-type metabolism.

It is unclear if the pituitary-steroid interaction participates in instances of sexually dimorphic induction of cytochrome P-450. PB treatment increased the N-demethylation of EM and 3,4-dimethyl aminobenzene to a greater extent in male rats than in females (El Defrawy and Mannering, 1974). The sexually differentiated response to PB was abolished by hypophysectomy (Finnen and Hassall, 1984), but this need not mean that pituitary secretions regulate xenobiotic induction as well as androgen directed sex differences. If PB induction of cytochromes P-450 was superimposed upon the different basal isozyme levels determined by the pituitary, it could yield an apparent sex difference in PB induction. The knowledge of cytochrome P-450 regulation at the cellular level is so limited that most explanations for this phenomenon would be highly speculative.

The influence of the pituitary in induction by MC-type compounds is also complex. MC did not alter sexually differentiated activities in intact females or hypophysectomized males or females, but reduced them in the intact male (Kato and Takayanaghi, 1966; Finnen and

Hassall, 1984). This suggested an antagonistic influence of MC or a metabolite on pituitary-mediated masculinization of monooxygenation. However, the magnitude of MC's effect on metabolism in hypophysectomized rats was less than in intact rats, regardless of whether or not the investigated reaction is sexually differentiated (Burke et al, 1978; Finnen and Hassall, 1984). This implies that part of the effect of hypophysectomy on MC induction is a general phenomenon unrelated to sex differences in metabolism.

Growth Hormone

It was postulated that the pituitary effected feminization by secreting a compound called "feminotropin." Pituitaries implanted into hypophysectomized female rats caused feminization of testosterone metabolism, irrespective of the donor's sex (Denef, 1974). Growth hormone (GH) or prolactin, both known to be secreted by ectopic pituitaries, were suggested to be possible feminizing factors. Experimentally, GH treatment altered drug metabolizing patterns in male rats toward feminine metabolism (Wilson, 1970). Hypophysectomized, adrenalectomized rats injected with GH demonstrated reduced concentrations of hepatic cytochrome P-450 and cytochrome c reductase activities (Wilson and Spelsberg, 1976). Kramer et al (1979) concluded that GH was more likely than prolactin to be feminotropin as even large amounts of prolactin have little or no effect on hepatic steroid metabolism (Gustafsson and Stenberg, 1975). Multiple injections or the continuous infusion of rat GH (rGH) or human GH (hGH) into male rats feminized patterns of hepatic metabolism (Kramer and Colby, 1976; Mode et al, 1981; Mode et al, 1982). Rat pituitaries were fractionated and assayed for feminotropin, and it was identified conclusively to be growth hormone (Mode et al, 1983).

Although GH was shown to be the feminizing factor, serum levels of GH were not significantly correlated with feminization of metabolism in normal rats. This appeared to be resolved by the demonstration of a sex dependent pattern of GH secretion in adult rats. In males, there were regular surges of plasma GH levels every 3-4 h with low, often undetectable levels between peaks, whereas in females the pattern was more variable and included sustained periods of low, almost continuous GH secretion (Eden, 1979; Clark et al, 1987). Increasing the frequency of hGH injections in both male and

female hypophysectomized rats increased the degree of feminization of hepatic steroid metabolism (Mode et al, 1981; Mode et al, 1982). The continuous infusion of GH resembles the female secretory pattern more than the male, explaining how it may impart feminization.

Cytochrome P-450 Isozymes

At least 13 hepatic cytochrome P-450 isozymes have been purified from rats, and they demonstrate a diverse capacity for substrate metabolism (Table 1-2). The sex differences in hepatic metabolism in rats are explicable, in part, by the presence of sex-specific forms of hepatic cytochrome P-450 (Thomas et al, 1981). Both male-specific and female-specific cytochromes P-450, present in insignificant amounts in the other gender, have been purified from untreated adult rats by several laboratories. Male specific P-450 RLM5 (Cheng and Schenkman, 1982) is also identified as 2c (Waxman, 1984), P-450h (Ryan et al, 1984), P-450 male (Kamataki et al, 1983) and P-450 UT-A (Guengerich et al, 1982). Immunoreactive P-450 RLM5 was detected in microsomes from adult males but not from immature males or adult females (Waxman, 1984). The amount of anti-RLM5 reactive material was decreased in microsomes by castration of male rats and was recovered with testosterone, and was induced by testosterone in ovariectomized females (Kamataki et al, 1983). Neonatal castration, hypophysectomy, or hGH infusion suppressed RLM5 levels in male rats, whereas hypophysectomy elevated RLM5 levels in females (Morgan et al, 1985a;b). Thus regulation of RLM5 is in accord with the regulation of monooxygenase activities (androgen 16α-hydroxylation) conducted by this isozyme.

Other male specific isozymes are P-450 RLM3 (Cheng and Schenkman, 1982), also known as P-450g (Ryan et al, 1984), and P-450 PCN-E (Guengerich et al, 1982), also known as PB-2a (Waxman, 1988). RLM3 is a strain-specific isozyme. P-450 PCN-E is male specific, but inducible in female rats by pregnenolone 16α-carbonitrile (PCN). Both isozymes conduct androgen 6β-hydroxylation, although PCN-E appears to be primarily responsible for this male-specific activity in normal adult rats (Waxman et al, 1985).

The sex specific isozymes may be pivotal in sexually differentiating steroid metabolism. Cytochrome P-450 15β (MacGeoch et al, 1984), isolated from hepatic microsomes of female rats, catalyzed

TABLE 1-2: RAT HEPATIC P-450 ISOZYMES

Designations	Characteristic Properties
P-450a; UT-F	Steroid hormone 7α-hydroxylase
P-450b; PB-B; PB-4	Major PB-inducible form; testosterone 16β-hydroxylase and drug hydroxylase
P-450c; BNF-B	Major MC-inducible form; aryl hydrocarbon hydroxylase
P-450d; ISF-G	Major isosafrole-inducible form
P-450e; PB-D; PB-D	97°/o homologous with P-450b; low activity
P-450f	Constitutive; crossreactive with P-450b
P-450g; RLM3	Male specific, strain-dependent testosterone 6β-hydroxylase
P-450h; UT-A; 2c; RLM5; male	Male-specific testosterone 2α/16α-hydroxylase and E_2 2-hydroxylase
P-450i; UT-I; 2d; 15β; female	Female-specific steroid disulfate 15β-hydroxylase
P-450j; RLM6	Ethanol-inducible nitrosamine demethylase
P-450k; PB-C; PB-1	PB inducible, hormone independent
P-450p; PB-2a; PCN-E	PCN inducible testosterone 6β-hydroxylase and E_2 2-hydroxylase
2a	Male-specific testosterone 6β-hydroxylase

From Waxman, 1988

the 15β-hydroxylation of androstanediol-3α,17β-disulfate, a female specific microsomal activity. This isozyme has been purified in other laboratories as P-450 2d (Waxman, 1984), P-450i (Ryan et al, 1984), P-450 female (Kamataki et al, 1983), and P-450 UT-I (Schenkman et al, 1987). The levels of immunodetectable microsomal P-450 15β were low in both male and females rats of up to 21 days of age, then increased in females while remaining low in males. Like the female-specific activity, the level of P-450 15β was decreased in female rats by testosterone treatment and hypophysectomy, but not by ovariectomy. However, P-450 15β levels were increased in hypophysectomized female rats by hGH. In normal male rats P-450 15β levels were low, but were elevated by hGH treatment or neonatal castration (Kamataki et al, 1983; MacGeoch et al, 1984; Waxman et al, 1985; Kamataki et al, 1985; MacGeoch et al 1985; Dannan et al, 1986a). Altogether, both sex steroids and GH secretion appear to regulate sex specific isozymes and subsequent sex specific monooxygenase activities by rat microsomes.

The sex differences in E_2 2-hydroxylase activity in untreated rats are readily accounted for by the sex-specific isozymes. This activity was greater in hepatic microsomes from male rats than from females, and the levels of activity were decreased by castration of male rats or testosterone treatment of females (Barbieri et al, 1978; Dannan et al, 1986b). The levels of microsomal activity were decreased in intact male rats by GH infusion or by testosterone treatment in females (Quail and Jellinck, 1987). Inhibition studies have suggested that male-specific RLM5 and PCN-E conduct E_2 2-hydroxylation in microsomes from untreated male rats (Dannan et al, 1986b). Reconstituted RLM5 was an efficient E_2 2-hydroxylase (Cheng and Schenkman, 1984), and the patterns of regulation of RLM5 and E_2 2-hydroxylation are similar. Although reconstituted PCN-E was not an efficient E_2 2-hydroxylase, both the isozyme and the enzyme activity were inducible by PCN (Guengerich et al, 1982; Dannan et al, 1986b), supporting the suggestion that this isozyme participates in microsomal E_2 2-hydroxylation as well. Possibly, E_2 2-hydroyxlase activity by PCN-E is lost during purification. It is apparent though, that sex differences in E_2 2-hydroxylation by rat microsomes result directly from sex differences in the levels of the responsible isozymes.

Review of Teleost Literature

The cytochrome P-450 monooxygenases in fish are similar in many respects to mammalian systems. The following discourse on teleost monooxygenases is based on reports by Chambers and Yarbrough (1976), James et al (1979), James and Bend (1980), and Stegeman (1981). Teleost monooxygenase activities require O_2 and NADPH, and are localized in the microsomal fractions of subcellular preparations. The liver contains most of the monooxygenase activity, although it has been detected to a lesser extent in several extrahepatic tissues. As will be discussed, many monooxygenase activities detected in mammalian microsomes are also present in teleost microsomes. A striking distinction from mammalian systems is a general lack of response to PB-type compounds, while many MC-type agents are effective inducers of the same monooxygenase activities that are induced by these compounds in mammals. In some cases induction in teleosts is accompanied by a downward shift of the CO-ligated spectrum, suggesting induction of isozymes with lower absorbance maxima than in the control group.

Teleost Sex Differences in Hepatic Monooxygenases

Xenobiotic metabolism: The research of hepatic mixed function oxidases and their regulation by hormonal factors does not have the scope in teleosts that it does in mammalian systems, but it appears that some of the patterns of steroid and xenobiotic metabolism resemble those seen in mammals. The sex differences in teleost monooxygenase activities are summarized in Table 1-3. As in mammals, these differences were not apparent until the teleosts had matured. In general, the specific content of hepatic microsomal cytochrome P-450 was higher in mature male teleosts than in females (Hansson and Gustafsson, 1981a; Stegeman and Woodin, 1984). In some instances, male teleosts resembled male rats by metabolizing foreign compounds faster than females. Specific ethoxycoumarin O-deethylase (ECOD) activity by vendace (Coregonus albula) and rainbow trout, p-nitroanisole O-deethylase (PNAO) activity by rainbow trout, and APDM activity by both rainbow trout and brook trout (Salvelinus fontinalis) were all greater in males than in females. Similar patterns of sex differences were observed for specific BP metabolism in male vendace, prespawning cunner (Tautogolabrus adspersus), and killifish (Fundulus heteroclitus) (Walton et al, 1978; Stegeman and Chevion, 1980;

TABLE 1-3: SEX DIFFERENCES IN TELEOST HEPATIC MICROSOMAL ACTIVITIES

Species	Microsomal Activity	Male vs. Female rates of activity
Rainbow trout		
	APDM, PNAO, ECOD per mg protein	male > female[1]
	APDM, PNAO per nmol P-450	male ≥ female[1]
	Benzo[α]pyrene hydroxylation per mg protein	male = female[1]
	Benzo[α]pyrene hydroxylation per nmol P-450	male ≤ female[1]
	Androstenedione, 6β-hydroxylation and 17-HSOR per mg protein	male > female[2]
	Androstenedione, 5α-reductase, 16-hydroxylation per mg protein	male = female[2]
Brook trout		
	APDM per mg protein	male > female[1]
	APDM per nmol P450	male = female[1]
	Benzo[α]pyrene hydroxylation per mg protein	male = female[1]
	Benzo[α]pyrene hydroxylation per nmol P-450	male ≤ female[1,3]
	Testosterone 16β-hydroxylation per nmol P-450	male < female[3]
Vendace		
	Benzo[α]pyrene hydroxylation, ECOD per mg protein	male > female[4]
Cunner		
	Aryl hydrocarbon hydroxylation per mg protein	male > female[5]
Killifish		
	Benzo[α]pyrene hydroxylation per mg protein	male > female[6]
	Benzo[α]pyrene hydroxylation per nmol P-450	male = female[3]
Winter flounder		
	EROD per nmol P-450	male > female[3]
	Testosterone 6β/16β-hydroxylation per nmol P-450	male < female[3]

[1]Stegeman and Chevion, 1980; Forlin, 1980; Koivusaari et al, 1981
[2]Hansson and Gustafsson, 1981a
[3]Stegeman and Woodin, 1984
[4]Lindstrom-Seppa, 1985
[5]Walton et al, 1978
[6]McKee et al, 1983

Lindstrom-Seppa et al, 1981; Stegeman and Woodin, 1984). 2,4-Diphenyloxazole, a BP substitute, was metabolized in vitro by prespawning males and postspawning male and female cunners 2-3 times faster than by prespawning female cunners. As the prespawning stage in fish corresponds to peak serum titers of sex steroids (Campbell et al, 1975; Wingfield and Grimm, 1977; Lambert et al, 1978), this suggested that steroids could be regulating xenobiotic metabolism.

Because of the sex differences in the specific content of P-450, rates of monooxygenase activities per nmol P-450 (i.e., the turnover values) did not always demonstrate the same sex pattern as the specific activity (see Table 1-3). BP hydroxylation in killifish was sexually differentiated per mg protein but not differententiated per nmol P-450 (Stegeman and Woodin, 1984). In rainbow trout and brook trout, specific BP metabolism was not sexually differentiated while activity normalized to cytochrome P-450 was in females greater than or equal to activity in males. Turnover values for APDM and PNAO activities in males were greater than or equal to levels in the female (Stegeman and Chevion, 1980; Forlin 1980). However, in winter flounder, EROD activity both per mg protein and per nmol cytochrome P-450 was greater in males than in females (Stegeman and Woodin, 1984).

Steroid metabolism: Steroid metabolism is also described in fish, although it is less extensively studied. Slices of liver from rainbow trout had 5α- and 5β-reductase and 3α-, 3β-, and 17-hydroxysteroid oxidoreductase (HSOR) activities (Lisboa and Breauer, 1966, cited in Ozon, 1972a). Hydroxysteroid oxidoreductases in the supernatant fraction from rainbow trout liver metabolized estrogens at C-16α, -16β, and -17β (Breauer, 1963, cited in Ozon, 1972b). Post-mitochondrial supernates from the hagfish demonstrated 5α-reductase and 17-HSOR activities, but one species (Myxine glutinosa) had 3α-HSOR activity while the other (Eptatretus burgeri) had 3β-HSOR (Inano et al, 1976; Hansson et al, 1979). Microsomes from dogfish (Squalus acanthias) and rainbow trout also showed 5α-reductase and 3α- and 17-HSOR activities (Hansson et al, 1979). Therefore, fish including teleosts have many of the steroid reductase activities documented in mammals. However, sex differences in A-ring reduction were not reported in teleosts, although this reductase shows higher levels of activity in female rats.

Teleost microsomes oxidized sex steroids at several positions. Inano et al (1976) observed only 7α-hydroxylation of testosterone by E. burgeri microsomes whereas Hansson et al (1979) observed only 6β-hydroxylation in M. glutinosa. The difference in site-specific metabolism may reflect the use of different hagfish genera, but both studies illustrate this primitive fish's limited capacity to hydroxylate androgens. Androstenedione was hydroxylated by dogfish microsomes at C-6β and -16β and by rainbow trout microsomes at C-1α, -6β, and -16β (Hansson et al, 1979). In vitro 6β- and 16β-hydroxylase activities were also documented in winter flounder, killifish, and brook trout (Stegeman and Woodin, 1984). Rainbow trout microsomes hydroxylated E_2 at C-6α, -6β, -7α, and -16β (Hansson and Rafter, 1983); catecholestrogen formation was not reported.

Microsomes from teleost species displayed sex differences in steroid metabolism, but these patterns do not always parallel those in rats (Table 1-1; 1-3). As stated, there was no sex difference in 5α-reductase activity in trout microsomes, although in rats it may be 3-10 times greater in females than in males. Specific androstenedione 6β-hydroxylase and 17-HSOR activities decreased in maturing female rainbow trout (Hansson and Gustafsson, 1981a). In winter flounder, testosterone 6β-hydroxylase activity normalized to cytochrome P-450 was 5-10 times greater in mature females than in males (Stegeman and Woodin, 1984). This contrasted with the greater turnover values for 6β-hydroxylase activity in male rats. 16β-Hydroxylation of androgens was greater in mature female winter flounder and brook trout than in males (Stegeman and Woodin, 1984). Since these sex differences were manifested in sexually mature fish whose plasma steroid titers are peaking, it suggested that the sex steroids, E_2, testosterone, and 11-ketotestosterone, may influence hepatic metabolism.

Steroid Regulation of Hepatic Monooxygenases

The focus of this study was the role of E_2, known to stimulate hepatic vitellogenesis, in regulating hepatic monooxygenase activity. Normally, circulating E_2 stimulates hepatic yolk protein synthesis, or vitellogenesis, in the adult female fish (Ho, 1987). Experimentally, E_2 treatment can initiate vitellogenesis in adult male and female fish and in immature fish. Liver weight, protein content and RNA content all increase, consistent with the stimulation

of protein synthesis (Emmerson et al, 1979; Haux and Norberg, 1985). Lipid content is increased while glycogen levels are decreased. On the ultrastuctural level, there is a proliferation of the rough endoplasmic reticulum and enlarged Golgi bodies (van Bohemen et al, 1982), as well as an unexplained decrease in microsomal protein per g liver (Stegeman et al, 1982). Serum levels of vitellogenesis increased in a dose-dependent manner following E_2 treatment, indicating synthesis of vitellogenin by the liver and secretion into the bloodstream (Ho, 1987).

Both androgens and estrogens induced changes in hepatic monooxygenase activities, but E_2 appeared to be the major regulatory steroid in teleosts whereas androgens are dominant in mammals. Some monooxygenase activities in juvenile teleosts were decreased by E_2 treatment while androgens were without effect (Forlin and Hansson, 1982; Hansson 1982; Hansson, et al, 1982; Stegeman et al, 1982). This may represent a major difference between teleosts and mammals in the regulation of cytochrome P-450 by sex steroids.

In some respects, trout responded to E_2 treatment with adult-like patterns of microsomal xenobiotic and steroid metabolism. E_2 administration suppressed microsomal cytochrome P-450 and rates of BP, p-nitroanisole, and EM metabolism in juvenile rainbow trout, if all were expressed relative to microsomal protein. However, E_2 treatment was without effect if monooxygenase activities were normalized to cytochrome P-450 (Forlin and Hansson, 1982). In immature brook trout, microsomal BP hydroxylation normalized to protein or cytochrome P-450 was not affected by E_2 or testosterone treatment (Stegeman et al, 1982). The apparent K_m for PNAO was slightly higher in maturing female trout than in juvenile fish (Forlin and Lidman, 1981), and was elevated in immature fish by E_2 treatment (Forlin and Hansson, 1982). In adult brook trout and rainbow trout, 6β-hydroxylation was greater in males than in females (Hansson and Gustafsson, 1981b; Stegeman and Woodin, 1984), and was suppressed in immature trout by E_2 administration (Hansson and Gustafsson, 1981a; Stegeman et al, 1982). This indicates that circulating E_2 may regulate monooxygenase activities in adult females as it did experimentally in juveniles.

The Pituitary

The pituitary is obligatory in effecting sex differences in microsomal metabolism in rats, but its role in regulating metabolism in teleosts is unclear. In a study utilizing immature rainbow trout, both hypophysectomy and testosterone administration increased 17-HSOR activity, and testosterone treatment of hypophysectomized fish increased this activity more than either regime alone. This suggested that there could be two channels regulating 17-HSOR activity, one which depended on the pituitary and one which did not. E_2 suppressed the testosterone-induced increase in 17-HSOR activity in intact juvenile rainbow trout (Hansson, 1982), but not the increased activity observed with hypophysectomy (Hansson and Gustafsson, 1981a). It would appear that E_2 acted on hepatic metabolism through the pituitary in this respect. In intact and hypophysectomized fish E_2 suppressed 6β-hydroxylase activity without affecting 16β-hydroxylation, but the effect on the specific content of cytochrome P-450 was difficult to interpret due to fluctuating basal levels (Hansson and Gustafsson, 1981b). If E_2 was indeed affecting metabolism in the absence of the pituitary, then, unlike regulation in rats, the pituitary is not always obligatory for E_2 regulation of hepatic metabolism in fish.

There is evidence for a hypothalamo-pituitary axis in fish which responds to circulating steroid titers to affect both the gonads and, to some extent, the liver. The hypothalamo-pituitary-gonad axis is well characterized in the literature and may provide insight about the functioning of the hypothalamo-pituitary-liver axis in fish. Briefly, the gonads, stimulated by gonadotropic hormone (GtH) released from the pituitary, synthesize and secrete the sex steroids that stimulate the physiological preparation for spawning. GtH secretion appears to be under the control of release- and release-inhibiting-factors located in the hypothalamus (L.W. Crim et al, 1976; J.W. Crim et al, 1978; Peter et al, 1984). Both E_2 and testosterone bind to the brain (Kim et al, 1978; Myers and Avila, 1980) and either steroid treatment can stimulate GtH accumulation within the pituitary, although testosterone may be converted to E_2 first by aromatase activity in the brain (Olivereau and Olivereau, 1979; Callard et al, 1981; L.W. Crim et al, 1981). Both steroids have inhibited GtH secretion by a negative

feedback effect in mature trout and goldfish (Carassius auratus) (Billard and Peter, 1977; Billard, 1978). A positive feedback effect of steroids on GtH secretion in fish has been suggested to occur within the prespawning stages (L.W. Crim et al, 1981; Gielen and Goos, 1984). Steroids have been hypothesized to affect GtH secretion through the hypothalamic regulatory factors and indirectly via catecholamines (Goos et al, 1987).

It is possible that the fish pituitary releases a feminizing factor to elicit the female-type of metabolism. Salmon pituitary extracts, but not salmon partially purified GtH, depressed hepatic cytochrome P-450 levels in immature brook trout (Pajor, 1982). In rats, sex-specific patterns of GH secretion feminize metabolism. There are no published reports of the effects of administered GH on teleost monooxygenase activity, and knowledge of the regulation of GH secretion in teleosts is limited. GH is found in the somatotrophic cells of the proximal pars distalis of the pituitary (Nagahama et al, 1981; Cook et al, 1983). There is definitive evidence for a hypothalamic GH release-inhibiting factor in teleosts (Grau et al, 1985), and meager evidence for a GH releasing factor in one teleost, the flounder (Platichthys flesus) (Hall and Chadwick, 1979). The somatotrophic cells appeared to be stimulated in Poecilia latipinna during vitellogenesis (Young and Ball, 1983) and in female silver eels (Anguilla anguilla) after E_2 treatment (Olivereau and Olivereau, 1979). The serum level of anti-carp GH immunoreactive material rose in preovulatory female white suckers (Catostomus commersoni) and remained high in spent (post-spawning) females, but the levels were relatively invariant in male suckers (Stacey et al, 1984). The regulation of teleost GH secretion, then, involves hypothalamic factors and may also be associated with circulating E_2. Specific binding sites for GH purified from tilapia (Oreochromis mossambicus) were detected in hepatic microsomal membrane fractions of trout and other teleost species (Fryer, 1979). In view of GH administration effecting sex differences in rats, it would be interesting to know the effects of teleost GH on hepatic monooxygenase activity in teleosts.

The role of estrogens in fish physiology needs to be elucidated, given its importance in reproduction and its proposed role in regulating steroid metabolism. Furthermore, the regulation of E_2

levels has not been fully explored, and while there is an extensive literature on its biosynthesis in teleosts, there is a dearth of information on its metabolism. The liver and gall bladder from jewel fish (Hemichromis bimaculatus) injected with [^{3}H]E_2 demonstrated a high uptake of the steroid, substantiating a hepatic elimination route. Livers from males had higher concentrations of polar E_2 metabolites than livers from females, suggesting that E_2 was metabolized more rapidly in the males (Myers and Avila, 1980). A study employing [^{3}H]E_2 also implicted hepatic disposal of the steroid in rainbow trout (Forlin and Haux, 1985). E_2 metabolism may be important in reproduction, as high circulating E_2 titers stimulate vitellogenesis and metabolism may regulate these titers. If E_2 affects hepatic metabolism, directly or through the pituitary, then the levels of the steroid may influence its own metabolism.

Cytochrome P-450 Isozymes

A few microsomal cytochromes P-450 have been purified and characterized from teleosts (Table 1-4). Cytochrome P-450 isozymes were isolated from β-naphthoflavone treated rainbow trout (Williams and Buhler, 1982; 1984), untreated scup (Klotz et al, 1983; 1986), and BNF-treated cod (Gadus morhua) (Goksoyr, 1985). In scup, cytochrome P-450E is the major hydrocarbon metabolizing isozyme and appears to be the major hydrocarbon inducible form. P-450E is immunochemically related to trout LM4b and cod P-450c, which are also hydrocarbon hydroxylases and appear to be inducible by MC-type compounds. Antibodies against these isozymes inhibited EROD activity in microsomes from the same species against which antibodies were generated as well as other teleost species (Goksoyr, 1987).

P-450E demonstrated little steroid metabolizing activity. Of the other isozymes purified from scup, reconstituted P-450A and P-450B metabolized testosterone at C-6β and C-15α, respectively, although identification of the latter product is only tentative (Klotz et al, 1986). Neither isozyme was a significant BP hydroxylase. P-450 fractions C and D were also partially purified from scup microsomes by Klotz (1983) and in reconstitution both produced several testosterone metabolites, although in small quantities. None have been positively identified. Steroid metabolism has not been reported for reconstituted trout LM2 and LM4b, or for cod P-450c.

TABLE 1-4: TELEOST HEPATIC P-450 ISOZYMES

Species	M_r	Characteristics
Scup		
P-450A[1]	52,700	Testosterone 6β-hydroxylase
P-450B[1]	45,000	Tentative testosterone 15α-hydroxylase
P-450E[2]	54,300	Aryl hydrocarbon hydroxylase; major PAH-inducible form
Rainbow trout		
LM2[3]	54,000	Aflatoxin activator; lauric acid hydroxylase
LM4b[4]	58,000	Aryl hydrocarbon hydroxylase; major PAH-inducible form; immunochemically related to scup P-450E
Cod		
P-450c[5]	58,000	Aryl hydrocarbon hydroxylase; major PAH-inducible form; immunochemically related to scup P-450E

[1] Klotz et al, 1986
[2] Klotz et al, 1983
[3] Williams and Buhler, 1983
[4] Williams and Buhler, 1984
[5] Goksoyr, 1985

General Aims of Research

The general aims of this project were to examine the regulation of hepatic microsomal metabolism by E_2, as well as metabolism of E_2 itself, in winter flounder and scup. These species were selected for several reasons. Winter flounder are abundant in local waters from September to May and can be maintained at our facilities. Their hepatic monooxygenase systems display large sex differences, including higher rates of testosterone metabolism in females (Stegeman and Woodin, 1984), in contrast with the pattern of higher rates found in male rats and trout. There is also an extensive background literature on the endocrinology, reproduction, and microsomal metabolism in the flounder family (Pleuronectidae). Similarly, scup are abundant in local waters and available both during and after spawning. There is also a wealth of background information, compiled in Dr. Stegeman's laboratory, with respect to the cytochrome P-450 systems in scup.

The following avenues of inquiry were addressed:

1. The regiospecificity of E_2 metabolism by teleost monooxygenases, and whether or not this activity was sexually differentiated in scup and winter flounder
2. The characterization of sex differences in microsomes from gonadally mature scup and winter flounder, both with respect to enzymatic activities and levels of specific P-450 isozymes.
3. Treatment of immature winter flounder with E_2 to determine if the hormone could effect sex differences in in vitro xenobiotic and steroid metabolism.

Previous research on the effects of steroids in teleosts have concentrated mainly on aspects of reproduction, but evidence that steroids are also important in regulating hepatic monooxygenase activities have prompted this study. The results of the investigation only begin to suggest how the cytochrome P-450 system is regulated by steroid hormones in teleosts. Information on estradiol's regulatory roles in winter flounder could provide a unique model of the feminizing factor. The pronounced sex difference in cytochrome P-450 and monooxygenase activity in winter flounder and the distinctive greater monooxygenase activity with testosterone in microsomes from females also favored this species as a model system to study steroid regulation of hepatic metabolism.

CHAPTER 2: PATTERNS AND REGULATION OF ESTRADIOL METABOLISM BY HEPATIC MICROSOMES FROM TWO SPECIES OF MARINE TELEOSTS

Preamble

The following publication details the investigation of E_2 metabolism and the verification of E_2 2-hydroxylase activity in teleost microsomes. The adaptation of a facile assay for E_2 2-hydroxylation (Bulger and Kupfer, 1983) to use with teleost microsomes is described. Investigations of the regulation of E_2 2-hydroxylase activity in scup by BNF and in both scup and winter flounder by gender are also presented. The final section of the publication discusses the metabolism of E_2 by reconstituted P-450A, a scup isozyme previously shown to metabolize testosterone.

The first step in the analysis of E_2 metabolism by teleost microsomes was to examine E_2 metabolites separated by HPLC. This method was initially selected based on my experience with HPLC and the need for high resolution separation of E_2 metabolites. One of the metabolites was isolated and identified by mass spectrometry as estrone, the product of C-17β-ol oxidation. The indication that 2-OH-E_2 was also a metabolite of E_2 led to the challenge of positively identifying it and quantifying its rate of formation. For these ends, both TLC and measurement of ^3H_2O release from [2-^{3}H]E_2 were employed. The product was shown to be 2-OH-E_2, and specific inhibitors indicated that its formation was mediated by microsomal cytochrome P-450.

The regulation of E_2 2-hydroxylase activity by exogenous factors was investigated by examining the activity in microsomes from BNF-treated scup. Previously, reconstituted scup P-450E, the major BNF-inducible form, demonstrated low levels of E_2 2-hydroxylase activity (Klotz et al, 1986). BNF treatment did not affect the rate of E_2 2-hydroxylation expressed per mg microsomal protein, but turnover values for the activity were decreased to half of the levels of control fish. Antibodies against P-450E did not inhibit activity in microsomes from control or BNF-treated scup. Altogether, this indicated that P-450E did not contribute significantly toward microsomal E_2 2-hydroxylation.

Levels of E_2 2-hydroxylase activity per mg protein were lower in microsomes from gonadally mature female scup and winter flounder than from males. Activity per nmol cytochrome P-450 were not sexually differentiated in either species. In the winter flounder, activity

per g liver and per g body weight was also lower in adult females than in adult males or in immature females. These data were incorporated into the larger study of sex differences in steroid and xenobiotic metabolism discussed in Chapter 3.

In Klotz et al (1986), reconstituted cytochrome P-450A, a testosterone 6β-hydroxylase, also exhibited some E_2 2-hydroxylase activity. Studies with anti-P-450A on the role of this scup enzyme and its winter flounder homolog in E_2 2-hydroxylation are reported and discussed in Chapter 3. The regulation of E_2 metabolism and other monooxygenase activities by E_2 was then investigated in winter flounder, and the results are presented in Chapter 4.

GENERAL AND COMPARATIVE ENDOCRINOLOGY 66, 256–265 (1987)

Patterns and Regulation of Estradiol Metabolism by Hepatic Microsomes from Two Species of Marine Teleosts[1]

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Estradiol (E_2) metabolites formed *in vitro* by microsomes from the marine teleosts winter flounder (*Pseudopleuronectes americanus*) and scup (*Stenotomus chrysops*) included at least seven products detected by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The least polar metabolite was shown to be estrone by chromatographic and mass spectrometric identity with authentic estrone. Chromatographic analyses coupled with dual-label experiments also indicated formation of the catecholestrogen 2-hydroxyestradiol (2-OH-E_2), which was the most prominent metabolite determined by TLC. Analysis of microsomal E_2 2-hydroxylase activity by measuring the specific release of ^3H_2O from [2-^{3}H]E_2 indicated that it is mediated by cytochrome *P*-450. E_2 2-hydroxylase activity normalized to microsomal protein was lower in females than in males for microsomes from both mature scup and winter flounder. Activity normalized to liver weight or body weight in female winter flounder was also lower than that in males. However, activity normalized to cytochrome *P*-450 content did not show sex differences in either species. E_2 2-hydroxylase activity per nanomole cytochrome *P*-450 was reduced in scup treated with β-naphthoflavone, which induces the hydrocarbon hydroxylase cytochrome *P*-450E. Studies employing reconstituted *P*-450E and microsomes preincubated with polyclonal antibodies against *P*-450E confirmed that this isozyme does not catalyze E_2 2-hydroxylase activity in microsomes. However, preliminary work with scup cytochrome *P*-450A suggests that it may be an E_2 2-hydroxylase. The studies establish that catecholestrogen formation is prominent in fish liver and that it is sexually differentiated, but further investigation is required to define the catalysts as well as the significance and regulation of this function.

The steroid hormone estradiol (E_2)[3] is the primary female reproductive hormone in vertebrates. In oviporous species including fish, E_2 initiates vitellogenesis, the synthesis of yolk proteins by the liver. E_2 function could depend on circulating levels of the hormone and its metabolism in various organs. The regulation of E_2 synthesis has been studied in many fish species (Fostier *et al.*, 1983), but other aspects of its metabolism are represented by very few studies in freshwater fish and none in marine species. Studies of jewel fish and rainbow trout injected with E_2 indicated elimination primarily via a hepatic route (Myers and Avila, 1980; Forlin and Haux, 1985). The hepatic cytochrome *P*-450 monooxygenase system is capable of metabolizing many steroids (Nebert and Negishi, 1982) and could be involved in oxidizing E_2 for elimination. In a single study, hepatic microsomes from rainbow trout metabolized E_2 to estrone and at least four hydroxylated products (Hansson and Rafter, 1983), and the formation of these hydroxylated metabolites appeared to be cytochrome *P*-450 mediated. However, 2-hydroxyestradiol (2-OH-E_2), the primary E_2 metabolite in mammals, was not among the identified metabolites in those reports

[1] Some of the results have appeared in preliminary form in *Fed. Proc.* (1986), 45, 575.

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[3] Abbreviations used: E_2, estra-1,3,5(10)-triene-3,17β-diol; BNF, β-naphthoflavone; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; EROD, ethoxyresorufin-*O*-deethylase.

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of hepatic E_2 metabolism by teleosts (Myers and Avila, 1980; Hansson and Rafter, 1983).

The studies reported here describe the first characterization of E_2 metabolism by hepatic microsomal systems in marine teleosts: the winter flounder (*Pseudopleuronectes americanus*) and scup (*Stenotomus chrysops*). Of particular interest is the observation of prominent 2-OH-E_2 formation by both species. Our results also indicate the regulation of this metabolism by endogenous factors. Winter flounder have demonstrated marked sex differences in the specific content of hepatic microsomal cytochrome *P*-450 and the metabolism of the nonsteroid substrate 7-ethoxyresorufin, indicating sexual differentiation in the regulation of some form(s) of cytochrome *P*-450 (Stegeman and Woodin, 1984). We determine whether E_2 2-hydroxylase activity is similarly regulated.

In addition, we evaluate regulation by exogenous factors, specifically foreign chemicals. In other studies, treatment with the cytochrome *P*-450 inducer β-naphthoflavone (BNF) caused a twofold increase in the amount of radiolabel in the bile of rainbow trout injected with [^{3}H]E_2 (Forlin and Haux, 1985). This corroborated a hepatic route for E_2 excretion in teleosts and suggested that elimination was stimulated by BNF. However, the individual biliary E_2 metabolites were not identified, and the nature of the effect of BNF on the profile of metabolites was not investigated. Understanding how E_2 is metabolized, and how this metabolism is affected by foreign compounds, would be useful in assessing the possible effects of xenobiotics on reproduction at the level of steroid hormones. Our results indicate that the rate of E_2 metabolism can be affected by BNF. Furthermore, the availability of purified cytochrome *P*-450 isozymes from scup and antibodies to one of these (Klotz *et al.*, 1983, 1986) enabled us to begin evaluating E_2 metabolism at the molecular level.

MATERIALS AND METHODS

Chemicals. [2-^{3}H]- and [4-^{14}C]E_2, 25.0 Ci/mmol and 57.0 mCi/mmol, respectively, were from New England Nuclear. 2-OH-, 4-OH-, 6αOH-, and 16αOH-E_2, E_2, and estrone were obtained from Research Plus (Bayonne, NJ) and Steraloids (Wilton, NH). Radiolabeled E_2 was diluted with unlabeled E_2 to the desired specific activity and purified by TLC. 7-Ethoxyresorufin was purified as described by Klotz *et al.* (1983). NADPH, activated charcoal, and dextran (clinical grade) were from Sigma. HPLC solvents were from Burdick & Jackson. All other solvents and reagents were of the highest quality commercially available.

Microsome and isozyme preparation. Winter flounder were collected from local waters by trawling, maintained in aquaria with flowing ambient seawater, and sacrificed within 2 days of capture. Scup were collected by angling and were either sacrificed within hours or maintained in aquaria with flowing seawater and fed a diet of squid and Purina Trout Chow. Unless noted otherwise, BNF-treated scup were injected intraperitoneally with approximately 15 mg BNF in corn oil and killed 5 days later. Microsomal fractions were prepared from excised livers and the cytochrome *P*-450 content was measured by difference spectroscopy as described elsewhere (Stegeman and Binder, 1979). Cytochrome *P*-450E, the major aromatic hydrocarbon inducible form, cytochrome *P*-450A, a major testosterone 6β-hydroxylase, NADPH-cytochrome *P*-450 reductase, and cytochrome b_5 were purified from scup liver by Klotz *et al.* (1983, 1986). Rabbit cytochrome b_5 was a gift from Dr. Y. Takagaki of the Massachusetts Institute of Technology. Rabbit polyclonal antibodies to *P*-450E were prepared by standard methods and their specificities have been described (Kloepper-Sams *et al.*, 1987).

Enzyme assays. Ethoxyresorufin-*O*-deethylase (EROD) activity was assayed as described by Klotz *et al.* (1983). Due to the lability of catecholestrogens, 2-OH-E_2 formation was routinely assayed by ^3H_2O release from [2-^{3}H]E_2 (Kupfer *et al.*, 1981). Our modified assay consisted of microsomes (0.1–0.3 mg/ml) or reconstituted scup isozymes (30 pmol isozyme, 1–2 equivalents scup or rabbit cytochrome b_5, 1–2 equivalents scup reductase, and 8 μg dilaurylphosphotidylcholine), 25 μ*M* [2-^{3}H]E_2 (16 μCi/μmol), 1 m*M* EDTA, and 0.3 m*M* NADPH in 100 m*M* sodium phosphate buffer, pH 7.4, in a final volume of 200 μl. The reaction was initiated with NADPH, progressed at 25° for flounder and 30° for scup for 30 min, and was terminated with 200 μl ice-cold 16 m*M* $CaCl_2$ to aggregate the microsomes. Samples were transferred to test tubes containing dextran-coated charcoal (pellets from 200 μl of 1% activated charcoal and 0.5% dextran in 10 m*M* Tris, pH 8.0), vortexed, shaken at 0–5° for 15 min, and centrifuged at 5000*g*. 200-μl aliquots of the supernatant were counted in a Packard Tri-Carb scin-

tillation counter. For assays conducted in the presence of anti-*P*-450E, microsomes were preincubated with antibody (60 μg IgG/pmol microsomal *P*-450), EDTA, and buffer on ice for 20 min before the substrate and co-factor were added.

HPLC and TLC. Microsomal estradiol metabolites were prepared for HPLC and TLC analysis by the method of Bulger and Kupfer (1983) except that the final reaction mixture contained 25 μ*M* E_2 and was incubated for 30 min at 30°. A dual-label experiment was performed in which microsomes were incubated with equimolar amounts of [4-^{14}C]E_2 (4.0 μCi/μmol) and [2-^{3}H]E_2 (28.0 μCi/μmol). Carbamazepine was added as an internal standard, and the reaction mixture was extracted three times in anhydrous ether, washed with 20% NaCl, and dried under N_2. The residue was dissolved in a small amount of acetonitrile, injected onto a DuPont 850 HPLC, and separated on a Rainin Microsorb C18, 4.6 mm × 25 cm, 5 μm ODS column using 25% acetonitrile in water for 10 min followed by 40% acetonitrile, at 1 ml/min, monitored at 285 nm. Fractions were collected every 0.8 min in Kimble Petite scintillation counting vials and dried overnight at 50°. The residue was resuspended with 3 ml Scintiverse II (Fisher Scientific Co.) and the vials were capped and counted.

Metabolites of [4-^{14}C]E_2 were also separated on silica TLC plates (Merck) that were impregnated with ascorbic acid to minimize catecholestrogen oxidation (Gelbke and Knuppen, 1972) and developed twice in ethyl acetate/hexane/acetic acid, 75:20:5. Autoradiography was accomplished by exposing the plate to Kodak X-Omat film for 10–14 days. A metabolite suspected to be 2-OH-E_2 was scraped from the plate, eluted from the silica with methanol, spotted onto TLC plates with 100 μg cold 2-OH-E_2, and developed in one of three solvent systems described in the text. Autoradiography of the plates was accomplished as described above.

Mass spectrometry. The metabolite to be analyzed by mass spectrometry was collected from the HPLC in chloroform-washed glassware, extracted with chloroform, and dried under N_2. The sample was resuspended in chloroform and subjected by probe distillation to electron impact ionization mass spectrometry on a Finnigan Model 4500. Crystals of authentic estrone were dissolved directly in chloroform and similarly analyzed.

RESULTS

Estradiol Metabolites

High-performance liquid chromatography of radiolabeled E_2 metabolites from scup microsomes (Fig. 1) resolved five peaks in addition to the parent E_2. Two of

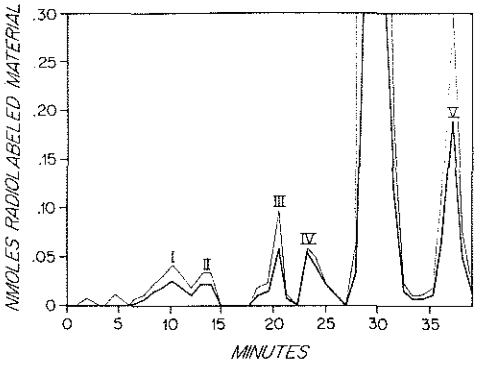

FIG. 1. Separation of dual-labeled E_2 metabolites by HPLC. Scup microsomes (0.2 mg) were incubated with [4-^{14}C]- and [2-^{3}H]E_2, and the metabolites were extracted and chromatographed as described under Materials and Methods. Metabolites are numbered in order of elution. The parent E_2 eluted after peak IV. The thick line shows the elution of ^{14}C-labeled material; the thin line shows the elution of total radiolabeled material.

the peaks eluting prior to E_2, designated I and II, had the same retention times as did 6αOH- and 16αOH-E_2 (estriol), respectively. However, these products have not been positively identified as yet. The third peak eluting prior to E_2, peak III, did not coelute with any of the standards. Peak IV co-chromatographed with authentic 2-OH-E_2, and is considered further below. Peak V, the major component seen on HPLC, eluted after E_2 and co-chromatographed with estrone. Mass spectrometry (Table 1) confirmed the identity of peak V as es-

TABLE 1
MASS FRAGMENTS OF AUTHENTIC ESTRONE AND ESTRADIOL METABOLITE FORMED BY SCUP LIVER MICROSOMES

Estrone		Metabolite	
m/z	Relative intensity	*m/z*	Relative intensity
146	31	146	32
172	26	172	26
185	35	185	35
213	18	213	18
270	100	270	100

trone. The elution pattern of metabolites from winter flounder microsomes were similar to patterns seen with metabolites from scup.

The possible identity of peak IV as 2-OH-E_2 was further evaluated in dual-label studies using [4-^{14}C]- and [2-^{3}H]E_2. HPLC analysis of products from the dual-labeled substrate (Fig. 1) showed that peak IV contained predominantly ^{14}C. The other peaks resolved by HPLC had ^{3}H:^{14}C ratios like those in the parent E_2, thus demonstrating no apparent ^{3}H release associated with formation of these metabolites. This selective loss from peak IV of ^{3}H from [2-^{3}H]E_2 could be associated with 2-OH-E_2 formation, thus further indicating the presence of E_2 2-hydroxylase activity.

Autoradiography of [4-^{14}C]E_2 metabolites from scup, separated on TLC (Fig. 2a), revealed at least seven distinct metabolites. The single spot that migrated further than E_2, thus being less polar than E_2, co-migrated with estrone, consistent with the findings from HPLC. The most prominent spot more polar than E_2 was suspected to be 2-OH-E_2, on the basis of its coincident migration with 2-OH-E_2. This metabolite was eluted from the silica and rechromatographed with unlabeled 2-OH-E_2 in three different solvent systems; the radiolabel co-migrated with the reference steroid in all three systems (Figs. 2b–d). A small amount of material migrated ahead of the 2-OH-E_2 in each of these TLC analyses and, in the solvent system corresponding to Fig. 2d, 4% of the label co-migrated with 4-OH-E_2 and 15% with the parent E_2. However, the majority of the labeled product migrated with authentic 2-OH-E_2. In Fig.

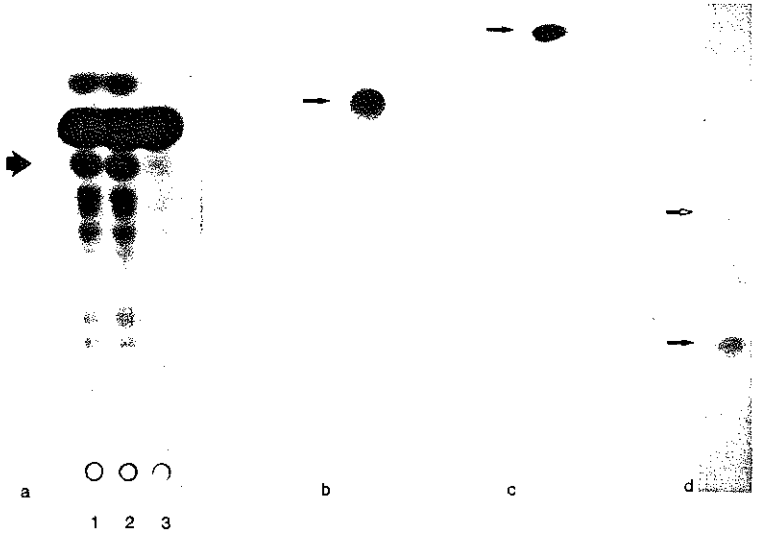

FIG. 2. Analysis of E_2 metabolites by TLC and autoradiography. (a) [4-^{14}C]E_2 metabolites from scup microsomes separated on ascorbic acid impregnated TLC plate and subjected to autoradiography as described under Materials and Methods. Lane 1, metabolites formed by control scup; lane 2, metabolites formed by BNF-treated scup; lane 3, blank reaction (minus co-factor). Position of authentic 2-OH-E_2 migration is marked by heavy arrow. (b–d) Putative 2-OH-E_2 eluted from plate (a) as described under Materials and Methods, and rechromatographed with cold authentic 2-OH-E_2 on plates developed twice in various solvent systems: in (b) ethyl acetate/hexane/ethanol, 80:15:5; in (c), ethyl acetate/hexane/acetic acid/ethanol, 72:13.5:10:4.5; in (d), chloroform/methanol/acetic acid, 96:3:1. In (b–d), position of authentic 2-OH-E_2 is marked by a dark arrow. In (d), migration of 4-OH-E_2 is marked with an open arrow.

2a this represented 34% of the total product resolved by TLC, and the amount formed corresponded to an activity of 0.135 nmol/min/mg microsomal protein.

The above results indicate that 2-OH-E_2 is the most prominent metabolite of E_2 formed by teleost liver microsomes, and further analysis of E_2 metabolism centered on formation of this product. The lability of 2-OH-E_2 renders suspect quantitative product analysis by chromatography. Consequently, ^3H_2O liberation from [2-^{3}H]E_2 was used to monitor E_2 2-hydroxylation in these studies. The release of ^3H_2O from [2-^{3}H]E_2 in incubations with liver microsomes showed time and temperature dependence consistent with enzymatic activity. Formation of ^3H_2O was also dependent on NADPH and was inhibited by both cytochrome *c* and CO, indicating that E_2 2-hydroxylation measured by ^3H_2O release was cytochrome *P*-450 mediated (Table 2).

Regulation of E_2 Metabolism

Estradiol 2-hydroxylase activity was prominent in hepatic microsomes from both winter flounder and scup. The activity per milligram protein was similar between the two species, although the estimated turnover number, i.e., the activity per nanomole *P*-450, was substantially greater in scup microsomes (Table 3). In both species, the activity per milligram protein was significantly greater in gonadally mature or ripe males than in gonadally mature or gravid females; there was nearly a threefold difference between mature male and mature female winter flounder. The activity in mature females was also less than that in gonadally immature females. However, E_2 2-hydroxylase activity expressed per nanomole cytochrome *P*-450 was not sexually differentiated between mature males and mature females of either species.

TABLE 2
REQUIREMENTS FOR ESTRADIOL 2-HYDROXYLASE ACTIVITY WITH MICROSOMES FROM SCUP

Conditions	Percentage E_2 2-hydroxylase[a]
Standard: open to air, 0.3 m*M* NADPH	100 ± 2
Minus NADPH	3
1.6 m*M* NADH	8 ± 1
1.6 m*M* NADPH	109 ± 4
1.6 m*M* NADPH + 100 μ*M* cytochrome *c*	12 ± 1
N_2:O_2, 80:20	101 ± 1
CO:O_2, 80:20	52 ± 3

[a] Activity is relative to standard conditions. 100% activity was 0.508 ± 0.011 nmol/min/nmol *P*-450. Microsomes from BNF-treated sexually immature scup were utilized.

E_2 2-Hydroxylase Catalyst

Preliminary studies (see Klotz *et al.*, 1986) suggested that two scup cytochrome *P*-450 isozymes, *P*-450A and *P*-450E, could hydroxylate E_2 at the C-2 position in reconstitutions. Cytochrome *P*-450E, the major hydrocarbon hydroxylating isozyme from scup, is the apparent major hydrocarbon-inducible *P*-450 isozyme (Klotz *et al.*, 1983; Kloepper-Sams *et al.*, 1987). The potential for this isozyme to catalyze microsomal E_2 2-hydroxylase activity was determined by assaying microsomes from BNF-treated scup, which had high levels of *P*-450E, and from control scup which had low amounts of *P*-450E. Microsomal E_2 2-hydroxylase activity expressed per milligram protein did not differ between these control and BNF-treated scup. However, the estimated turnover values for E_2 2-hydroxylase (activity per nanomole cytochrome *P*-450) were significantly lower in microsomes from BNF-treated scup than in those from control scup (Table 4). By comparison, EROD activity, which is catalyzed almost exclusively by *P*-450E (Kloepper-Sams *et al.*, 1987), is strongly induced by BNF treatment (Table 4).

This indication that induced cytochrome *P*-450E did not catalyze microsomal E_2 2-hydroxylase activity was confirmed by im-

TABLE 3
ESTRADIOL 2-HYDROXYLASE ACTIVITY[a] IN MICROSOMES FROM SCUP AND WINTER FLOUNDER

Species	Mature male	Mature female	Immature female
Scup			
Units/mg	0.362 ± 0.141 (5)[b,c]	0.157 ± 0.037 (6)	
Units/nmol *P*-450	0.608 ± 0.142	0.889 ± 0.224	
Winter flounder			
Units/mg	0.296 ± 0.079 (13)[d]	0.089 ± 0.037 (13)[f]	0.224 ± 0.083 (6)
Units/nmol *P*-450	0.246 ± 0.096	0.343 ± 0.192	0.257 ± 0.058
Units/g liver	4.7 ± 1.8[d]	1.5 ± 0.8[f]	5.0 ± 2.3
Units/g body wt	0.063 ± 0.040[e]	0.038 ± 0.023[g]	0.069 ± 0.027

[a] Determined by ^3H_2O release from [2-^{3}H]E_2.
[b] Mean ± SD (*N*). Units are nmol 2-OH-E_2 produced per minute. Mature males and females significantly different, [c]$P = 0.05$; [d]$P < 0.001$; [e]$P < 0.05$. Mature and immature females significantly different, [f]$P < 0.01$; [g]$P < 0.05$. Data analyzed by Mann–Whitney *U* test.

munochemical analysis. Polyclonal antibodies to cytochrome *P*-450E did not affect E_2 2-hydroxylase activity in either control or induced microsomes (Fig. 3). By contrast, these antibodies inhibit better than 90% of BNF-inducible aryl hydrocarbon hydroxylase and EROD activities in scup microsomes (Kloepper-Sams *et al.*, 1987).

In initial reconstitution studies, cytochrome *P*-450A, also purified from scup liver, demonstrated E_2 2-hydroxylase activity that was substantially greater than that of *P*-450E (Klotz *et al.*, 1986). However, repeated reconstitutions of E_2 2-hydroxylase activity with the same preparation of *P*-450A showed diminishing activity, from 0.32 nmol/min/nmol *P*-450A to undetectable levels. This result suggests degradation of the stored isozyme, implying that the estimated turnover number previously reported for reconstituted *P*-450A could be substantially lower than the actual value. By contrast, repeated reconstitutions of E_2 2-hydroxylase activity with *P*-450E were low (0.04–0.09 nmol/min/nmol *P*-450E) while aryl hydrocarbon hydroxylase activity reconstituted by *P*-450E remained high.

TABLE 4
ESTRADIOL 2-HYDROXYLASE AND EROD ACTIVITIES IN MICROSOMES FROM CONTROL AND β-NAPHTHOFLAVONE-TREATED SCUP[a]

Activity	Control (*N* = 4)	BNF-treated (*N* = 4)
E_2 2-hydroxylase		
nmol/min/mg	0.250 ± 0.061	0.206 ± 0.062
nmol/min/nmol *P*-450	1.05 ± 0.113	0.498 ± 0.083[b]
EROD		
nmol/min/mg	0.31 ± 0.14	2.75 ± 1.00[b]
nmol/min/nmol *P*-450	1.27 ± 0.39	6.80 ± 2.17[b]

[a] Scup were injected intraperitoneally on Days 1 and 4 with 20 mg/kg BNF in corn oil and killed on Day 8.
[b] $P < 0.001$. Data analyzed by Student's *t* test.

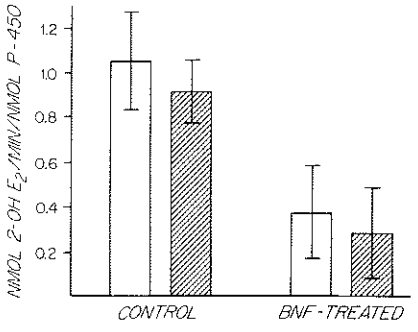

FIG. 3. The effects of normal IgG and anti-*P*-450E IgG on E_2 2-hydroxylase activity by microsomes from control and BNF-treated scup. Microsomes were preincubated with normal IgG (open columns) or anti-*P*-450E (shaded columns) for 20 min at 4° prior to assay.

DISCUSSION

The results here demonstrate that the catecholestrogen 2-OH-E_2 is the major E_2 metabolite formed by teleost liver microsomes, followed by estrone. In a study of E_2 metabolism by rainbow trout microsomes, estrone appeared to be the dominant metabolite, and the two major hydroxylated metabolites were reported as 7αOH- and 16αOH-E_2 (Hansson and Rafter, 1983). However, the experimental conditions in that study were such that catecholestrogens could have been autooxidized and thus undetected. The lability of catecholestrogens necessitated several approaches to verify formation of 2-OH-E_2. Analysis by both HPLC and TLC indicated that 2-OH-E_2 was a metabolite of E_2 and therefore that loss of ^3H from [2-^{3}H]E_2 was indicative of enzymatically mediated E_2 2-hydroxylation. This indirect method of detecting E_2 2-hydroxylase activity by measuring ^3H_2O release from [2-^{3}H]E_2 yielded rapid, reproducible results, facilitating demonstration that it was mediated by cytochrome *P*-450.

It is possible that E_2 4-hydroxylase activity could also cause ^3H_2O release from [2-^{3}H]E_2, via tautomerization of the product 4-OH-E_2 (Namkung *et al.*, 1985). With rat hepatic microsomes, 4-OH-E_2 is such a minor metabolite relative to 2-OH-E_2 (Ball and Knuppen, 1978) that release of ^3H from the 4-OH-E_2 would not significantly influence accurate measurement of E_2 2-hydroxylase activity. Since the bulk of radiolabeled material co-migrated with 2-OH-E_2 on a TLC plate developed in a system that cleanly separated 2-OH-E_2 and 4-OH-E_2 (Fig. 2d), it is assumed that 4-OH-E_2 production by teleost hepatic microsomes is also likely to be insignificant. Nevertheless, values referred to as E_2 2-hydroxylase activity in this discussion might be elevated by unrecognized E_2 4-hydroxylase activity.

Catecholestrogens are important products of mammalian estrogen metabolism as they can covalently bind to macromolecules and inhibit the enzymatic methylation of catecholamines (epinephrine and norepinephrine) *in vivo* and *in vitro* (Ball and Knuppen, 1979). They also possess both estrogenic and antiestrogenic properties in mammals (Ball and Knuppen, 1979). This is the first demonstration of catecholestrogen formation by teleosts. Other investigators have suggested but have not shown data for such activity in other organs (Lambert and van Oordt, 1982; de Leeuw *et al.*, 1985), and have hypothesized a participative role for it in the regulation of releasing gonadotropic hormone. Possibly, 2-OH-E_2 produced by the liver could affect this regulation or have other biological significance, but this is speculative.

In rats, sex differences in microsomal steroid metabolism, including E_2 2-hydroxylation, are attributable to the sex-specific regulation of steroid-metabolizing cytochrome *P*-450 isozymes (Waxman *et al.*, 1985; Dannan *et al.*, 1986). There are reports of sex differences in teleost steroid metabolism that appear to be hormonally regulated as well. Thus, microsomal 6β-hydroxylation of androstenedione and testosterone is lower in maturing female rainbow trout than in males. 6β-Hydroxylase activity can also be suppressed by E_2 treatment in juvenile rainbow trout and brook trout (Hansson and Gustafsson, 1981; Stegeman *et al.*, 1982; Hansson, 1982). Testosterone 6β-hydroxylation also appears to be lower in female than in male winter flounder (Snowberger and Stegeman, unpublished observation). Consistent with this, E_2 2-hydroxylase activity was similarly differentiated (Table 3).

Although E_2 2-hydroxylase activity expressed per milligram microsomal protein was sexually differentiated in mature fish, it is not clear that this involves specific regulation of the E_2 2-hydroxylase catalyst. That activity relative to microsomal cytochrome *P*-450 was unchanged (Table 3)

suggests that the efficiency of the cytochrome *P*-450 population for E_2 metabolism was not reduced in mature females. The livers of maturing and mature female fish synthesize large quantities of the protein vitellogenin, which appears to be rapidly secreted into the bloodstream in teleosts (van Bohemen *et al.*, 1982; Selman and Wallace, 1983). If microsomes from mature females were enriched by vitellogenin precursors or other newly synthesized proteins, these proteins might "dilute" the E_2 2-hydroxylase catalyst and thereby decrease its specific activity. However, E_2 2-hydroxylase activity normalized to liver weight or to body weight was lower by nearly one-half in gravid female winter flounder compared with that in sexually immature females. Furthermore, the microsomal cytochrome *P*-450 content per gram liver showed the same trend (data not shown). This suggests that there is suppression of a major fraction of microsomal cytochrome *P*-450, including but not restricted to the E_2 2-hydroxylase catalyst, in female flounder as they mature sexually.

It has been suggested that lower rates of E_2 metabolism in maturing female fish could contribute toward the increase of plasma E_2 that occurs before spawning (Hansson and Rafter, 1983). Lower activity per gram liver weight or per gram body weight in female fish, such as reported here, would support this, but the relationship between microsomal activity normalized to body weight and the actual activity in the fish is unclear. If the extrapolation does approximate catalytic activity in the whole animal, then suppression of the microsomal cytochrome *P*-450 catalyzing E_2 2-hydroxylase could decrease overall E_2 metabolism. The extent to which changes in hydroxylation as opposed to conjugation capacity would influence plasma E_2 levels is yet to be established.

A possible relationship between E_2 metabolism and circulating steroid levels presents an avenue whereby external factors, such as xenobiotics, could influence reproduction. Effects of xenobiotics on reproduction are known (von Westernhagen *et al.*, 1981; Sangalang *et al.*, 1981) but they are not completely understood. Halogenated hydrocarbons that can induce cytochrome *P*-450 reportedly produce lower androgen and estrogen levels in various species (Sivarajah *et al.*, 1978; Truscott *et al.*, 1983), suggesting that xenobiotics could act in part by affecting the levels of circulating steroid hormones. Although *in vitro* and histological studies with gonadal tissue (Freeman and Idler, 1975; Sangalang *et al.*, 1981) suggested alterations in steroidogenesis of exposed fish, elevated hepatic monooxygenase activity could induce steroid metabolism and elimination. Consistent with this, BNF-treated rainbow trout injected with [^{3}H]E_2 had higher amounts of E_2 metabolites in the bile relative to control fish (Forlin and Haux, 1985). Whether that effect resulted from changes in oxidative metabolism or changes in glucuronidation is not clear.

BNF is known to induce cytochrome *P*-450E, a scup isozyme that has some, albeit slight, E_2 2-hydroxylase activity in reconstitution. However, the fact that anti-*P*-450E did not inhibit microsomal E_2 2-hydroxylation in control or induced samples (Fig. 3), nor did it inhibit testosterone 6β-hydroxylation (Klotz *et al.*, 1986), establishes that *P*-450E does not contribute to these microsomal steroid hydroxylase activities. Rather, our results demonstrate that exposure to one type of cytochrome *P*-450 inducer, BNF, reduced the efficiency of E_2 2-hydroxylase turnover in scup (Table 4). The lower turnover for 2-OH-E_2 formation by BNF-induced microsomes is consistent with induction of isozymes less active toward E_2.

In contrast to scup *P*-450E, reconstituted scup *P*-450A had a high level of testosterone 6β-hydroxylase activity (Klotz *et al.*, 1986), prompting speculation that it was an important steroid metabolizing iso-

zyme. However, the apparent loss of catalytic function of *P*-450A in storage indicates that its capacity to metabolize E_2 cannot yet be fully compared with other isozymes. Despite this, the relatively high level of E_2 2-hydroxylase in even degraded samples of *P*-450A, and the high testosterone 6β-hydroxylase activity in earlier reconstitutions, reinforces the opinion that cytochrome *P*-450A may be a major steroid-metabolizing isozyme in scup. Further studies on the significance and regulation of E_2 metabolism by cytochrome *P*-450A are in progress.

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CHAPTER 3: SEX DIFFERENCES IN MONOOXYGENASES IN MATURE SCUP AND WINTER FLOUNDER

Introduction

In temperate waters, such as around Woods Hole, most fish species progress through an annual reproductive cycle. Sex differences in monooxygenase activity were investigated in the prespawning phase when the females were vitellogenic, i.e., a period of high hepatic biosynthetic activity. The winter flounder is a winter and early spring breeder, and around Cape Cod the spawning season begins in January with peak spawning occurring in February and March (Bigelow and Schroeder, 1953). Scup in these waters spawn in early summer, with the peak being in June (Morse, 1978). To assess the hepatic monooxygenase activity of gonadally mature individuals, winter flounder and scup were collected prior to and during their respective peak spawning seasons.

To fully characterize the patterns of sexually differentiated monooxygenase activity, it was advantageous to analyze the metabolism of several substrates. The choice of enzymatic assays was directed by previous indications of their being sexually differentiated in teleosts and by an interest in the metabolism of possible regulatory factors. Aminopyrine, 7-ethoxyresorufin, testosterone, and E_2 were selected as substrates because of previous reports of sex differences in their rates of oxidation in teleost microsomes (Stegeman and Kuhnhold, unpub. obs.; Stegeman and Woodin, 1984; Snowberger and Stegeman, 1987). Furthermore, in rats this activity is greater in microsomes from males than from females (Jellinck and Lucieer, 1965) while in humans the rate of oxidation appears greater in women than in men (Fishman et al, 1980). E_2 may also be an important regulator of hepatic cytochrome P-450 in fish (Stegeman et al, 1982; Hansson, 1982), and it has been suggested that plasma E_2 levels may be affected by the rate at which it is catabolized (Hansson and Rafter, 1983; Baroiller et al, 1987).

To determine if the levels of specific P-450 isozymes were sexually differentiated, teleost microsomes were subjected to quantitative immunoblotting with antibodies against purified scup isozymes. Previously described monoclonal antibody 1-12-3 against scup P-450E, the major aryl hydrocarbon hydroxylase in this species, recognized P-450E in scup microsomes and its homolog in winter flounder microsomes (Park et al, 1986; Kloepper-Sams et al, 1987).

P-450A, an apparent testosterone 6β-hydroxylase purified from scup microsomes, was also to be immunoquantified, but there was insufficient P-450A available to generate antibodies. Repeated attempts to purify native P-450A by standard chromatographic methods were unsuccessful. Therefore both P-450A and P-450B, another previously purified scup isozyme, were isolated from polyacrylamide gels and used to immunize rabbits. The isolation of these antigens and the characterization of the resultant antibodies will be described.

The immunoquantified levels of isozymes in scup and winter flounder microsomes were differentiated by sex and were correlated with enzymatic activity. A general pattern was observed in which sex differences in monooxygenase activities were linked to sexually differentiated levels of the responsible isozymes. Upon gonadal maturation, patterns of monooxygenases in female fish were altered while isozymes and activities in males appeared to change little compared to immature fish. The resultant sex differences were in some respects qualitatively similar to those in rats, but in teleosts altering enzyme levels in the adult female rather than in the male represented a unique form of monooxygenase regulation.

Materials and Methods

Fish Collection and Treatment

A sample of gonadally mature scup was collected by angling in Falmouth Harbor, MA, in mid-June 1982. A second sample of gravid scup was taken in mid-May, 1987 from standing nets in Nantucket Sound offshore from the Wianno Beach in Osterville, MA. Scup were also collected by angling in September-October for use as samples of gonadally immature scup or for purification of cytochrome P-450A and P-450B. Gonadally mature winter flounder were collected throughout January, 1983 by otter trawl from Menemsha Bight, Vineyard Sound. A second set of winter flounder was collected from Buzzards Bay in November, 1985, and were included in some of the analyses. Spawning and non-spawning killifish were obtained by seining in Little Sippewissett Marsh. Immature rainbow trout were obtained from the Sandwich Fish Hatchery, Sandwich, MA.

All animals except for the scup collected in May were sacrificed within one day of capture, and the livers were immediately processed to obtain microsomes. Livers from May scup were excised immediately

after capture and stored in liquid nitrogen until processing into microsomes 2-3 days later. In our experience, livers can be stored in liquid nitrogen for up to 2 months without compromising the quality of the microsomes.

Gonadally regressed winter flounder were also collected from Menemsha Bight in May 1985 and used to verify the induction of a cytochrome P-450E homolog in this species. Prior to treatment, these fish were maintained for 1 month on clean sediment in flow-through water of ambient temperature (18°C) in the Shore Laboratory at WHOI. Fish were maintained on a diet of chopped squid fed ad libitum. Fish were injected intraperitoneally with 20 mg/kg BNF in corn oil or corn oil alone on days 0 and 3 and were killed on the seventh day after initial injection; livers were removed and microsomal preparations were obtained as below.

Chemicals

[4-^{14}C]Testosterone (50 mCi/mmole) was obtained from New England Nuclear and purified by thin layer chromatography (TLC) (Waxman et al, 1983). [2-^{3}H]Estradiol was obtained and purified as described in Snowberger and Stegeman (1987). Androstenedione, 2α-, 2β-, 6β-, 7α-, 11α-, 14α-, 15α-, 16α-, and 16β-OH-testosterone standards were from Steraloids (Wilton, NH), Research Plus (Bayonne, NJ), or the Medical Research Council Steroid Reference Collection. Aminopyrine was obtained from Aldrich and was twice recrystallized from ethanol. 7-Ethoxyresorufin was purified as described by Klotz et al (1984). All other reagents were of the highest grade commercially available and were used without further purification.

General Methods

UV-visible spectra were obtained on a Cary 118C or Shimadzu UV-260 spectrophotometer. Discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Immunoblots were performed as in Kloepper-Sams et al (1987), except that immediately after transfer the nitrocellulose was temporarily stained with Ponceau S (Sigma) to visualize protein. The stain was completely removed during the blocking step of immunoblotting. Developed immunoblots were scanned with a Helena Labs Quick-Scan R and D densitometer (courtesy S. Watson, Associates of Cape Cod). Radioactivity of samples was determined with a Beckman LS

1801 or a Packard Tri-Carb 4000 Series liquid scintillation counter. Regression curves, correlations, and ANOVA statistical analyses were calculated with Statview (BrainPower, Inc.). If differences between means were found in the ANOVA analyses, Student's t test or Mann-Whitney U tests were employed to determine where the differences lay (Zar, 1974).

Microsomes, Enzymes, and Antibodies

Hepatic microsomes were prepared by sub-cellular fractionation of liver homogenates as before (Stegeman et al, 1979). Microsomal protein was determined by the method of Lowry et al (1951) or by Smith et al (1984) with bicinchoninic acid, using bovine serum albumin as a standard. Cytochromes P-450 and b_5 were quantified by difference spectroscopy (Stegeman et al, 1979).

Scup P-450A, P-450B, P-450 fraction C, P-450 fraction D, and P-450E were isolated from scup microsomes by Klotz et al (1983; 1986). Monoclonal antibody (MAb) 1-12-3 against scup P-450E was characterized as described elsewhere (Park et al, 1986; Kloepper-Sams et al, 1987). Polyclonal anti-trout LM2 IgG was a gift from Dr. D. Williams, Oregon State University. Anti-killifish vitellogenin antiserum was a gift from Dr. K. Selman, U. of Florida.

Microsomal Assays

NADPH-cytochrome P-450 reductase activity was measured by the reduction of cytochrome c (Binder and Stegeman, 1979). Ethoxyresorufin O-deethylase (EROD) activity was assayed as described by Klotz et al (1984). Aminopyrine N-demethylation (APDM) was assayed by measuring the rate of formaldehyde formation (Binder and Stegeman, 1979). E_2 2-hydroxylation was measured as described in the previous chapter (Snowberger and Stegeman, 1987).

Microsomal testosterone metabolism was assayed by the method of Waxman et al (1983), as modified by Klotz et al (1986). 12.5 pmole of [4-^{14}C]testosterone (25 μCi/μmole) in benzene/ethanol was dried under N_2 in a test tube, then redissolved in a small volume of methanol. Microsomes from scup or winter flounder (100 pmol spectral P-450) or a blank of boiled microsomes was added with buffer (50 mM Tris-Cl, pH 7.4) to 0.45 ml. The samples were preincubated at 30°C, and the reaction was initiated by adding 50 μl 5.0 mM NADPH. Aliquots of each 0.5 ml reaction mixture were sampled to determine total

testosterone levels. The reaction was terminated at 25 minutes with 0.5 ml cold acetone and extracted with 2 x 1 ml ethylacetate. The organic phase was dried under N_2 and spotted onto silica gel TLC plates impregnated with a fluorescent dye (Merck). Unlabeled metabolite standards were included with each lane. Plates were developed once in CH_2Cl_2/acetone, 4: 1, then twice in $CHCl_3$/ethyl acetate/ethanol, 70: 18: 12. Between developments, plates were dried horizontally for 30 minutes at room temperature.

Standards was visualized under a 254 nm lamp. The order of migration, from greatest to least distance from the origin, was androstenedione, testosterone, 2α-, 2β-, 16β-, 6β-, 11β-, 7α-, 14α-, 16α-, and 15α-OH-testosterone. Radiolabeled metabolites were located by exposing the TLC plate to Kodak X-Omat film for 3-7 days. Radioactivity was quantified either by scraping and counting each spot, or by scanning each lane for radioactivity with a Berthold TLC-Linear Analyzer (courtesy of J. Clark, U. Mass., Amherst). The quantity of metabolite formed was determined by dividing the radioactivity in a single spot by the total radioactivity in the lane, then multiplying by the total testosterone level in the initial reaction.

P-450A and P-450B: Isolation and Antibody Production

Because reconstituted scup P-450A metabolized testosterone at the C-6β position (Klotz et al, 1986), the production of antibodies against P-450A was a major goal for further studies of steroid metabolism. The purification of P-450A from scup microsomes to use as antigens was undertaken by the procedure outlined in Klotz et al (1986). The earlier results in purification were not replicable, and column fractions which should have contained essentially pure P-450A showed P-450A (52.7 kD), P-450B (45 kD), and other contaminants on SDS-PAGE analysis. Replacing the column resin and modifying the column buffers did not enhance the separation of P-450A from P-450B nor the elimination of extraneous proteins. Therefore, it was decided to isolate cytochrome P-450A by electroeluting it from SDS-PAGE. Although denatured, the electroeluted P-450A would still be suitable for immunizing rabbits. In addition, P-450B could be eluted from the same gel as P-450A and also injected into rabbits. Previously, reconstituted P-450B metabolized testosterone to several metabolites including apparent 15α-OH-testosterone. Through electroelution, it

would be possible to obtain anti-P-450B as a bonus in the acquisition of anti-P-450A.

Partially purified microsomes were used for electroelution. These microsomes were detergent solubilized, precipitated with 5-20^{o}/o polyethylene glycol, and chromatographed twice on DE-52 columns as described (Klotz et al, 1986). The pooled P-450A/B fraction was concentrated to approximately 1.5 nmol P-450/ml with an Amicon ultraconcentrator fitted with a PM-10 membrane. Approximately 5 ml was subjected to electrophoresis on slab gels that were 3 mm rather than 1.5 mm thick. The gels ran overnight and were water-cooled. Strips from the outer edges and center of the gel were Coomassie stained to determine the R_f's of P-450A and P-450B in order to locate the isozymes in the unstained panels. The P-450A and P-450B from the unstained panels were excised with a scalpel, diced into small cubes and distributed among sample cups of an Isco Electroeluter. The sample cup membranes had a 3000 dalton cutoff and there were 2-3 cups per buffer tank. The inner compartment and sample cup buffers were 0.001^{o}/o SDS in 0.46 mM Tris and 3.54 mM glycine, pH 8.4. The outer compartment buffer was 0.001^{o}/o SDS in 6.9 mM Tris and 53.1 mM glycine, pH 8.4. Buffer composition was a dilution of the electrophoresis running buffer, as suggested by the manufacturer. SDS was included to facilitate the migration of membrane-bound proteins from the polyacrylamide (Bhown et al, 1980). Electroelution was conducted at 1 watt for 4 hours or more, during which the buffer tanks were water-cooled. The eluted proteins were pipetted with care from the concentrating compartment of the sample cup. Purity was validated by electrophoretic analysis of 5 µg of each electroeluted sample on SDS-PAGE.

The two passages through DE-52 columns and the co-migration with standards strongly suggested that the isolated electroeluted proteins were P-450A and P-450B. To confirm identification, electroeluted proteins and previously purified P-450A and P-450B were subjected to proteolytic mapping by the method of Cleveland et al (1977), as modified by Waxman and Walsh (1983). Samples were diluted to ca 0.14 mg/ml and dialyzed extensively against buffer (0.125 M Tris-Cl, pH 6.7, containing 15^{o}/o glycerol). 40 µl of the dialyzed protein was mixed with 5 µl 2.5^{o}/o SDS and incubated in a boiling water bath for

one minute. Solubilized proteases were added in 5 µl aliquots to final concentrations of 5 and 20 ug/ml Staphylococcus aureus V8 protease and 1 and 5 ug/ml α-chymotrypsin (Sigma). Digestion proceeded for 90 minutes at 37°C and was terminated with 30 µl dye/denaturant (final concentration: 10% β-mercaptoethanol, 0.5% SDS and 0.015% bromophenol blue). Digestions were analyzed by SDS-PAGE on 13.5% polyacrylamide separating gels with a long (3.5 cm) stacking gel. Gels were silver-stained (Bio-Rad) according to manufacturer's instructions.

200 µg each of eluted P-450A and P-450B was mixed with Freund's complete adjuvent (Difco) at an adjuvent/antigen ratio of 2: 1. Four male New Zealand white rabbits were injected subcutaneously with emulsion containing 100 µg P-450A or P-450B. Each rabbit was boosted four weeks later with 100 µg of the same antigen and bled from the ear to assess antibody production. Thereafter, rabbits were bled at 10 day intervals. Rabbit IgG was purified from serum as described previously (Kloepper-Sams et al, 1987), and tested by immunoblot and Ouchterlony double diffusion on agarose plates (Thomas et al, 1976) for reactivity with P-450A and P-450B.

Results

Enzymatic Activities in Mature Scup and Winter Flounder

Basic sex differences: The sexually mature scup and winter flounder were distinguished from their gonadally immature counterparts by elevated gonadosomatic indices (Table 3-1). In mature fish of both species the testes were creamy colored and milt-filled, and the ovaries were pale orange and distended with eggs. The GSI of both sexes of scup collected in mid-May from Nantucket Sound were higher than for the scup collected in mid-June from Falmouth Harbor. This is consistent with a literature report that maximal GSI in scup is observed in May and decreases thereafter (Morse, 1978). In contrast, the gonads of immature scup collected in September were reduced such that the GSI of either sex was less than 0.3 (not shown). Similarly, the GSI of gonadally mature winter flounder was strikingly elevated over immature fish. The GSI of mature female winter flounder was approximately 9 in November and nearly double that in January. In mature male winter flounder the GSI ranged from 6 to 10 and was not different between the two samplings. The gonads of immature winter

TABLE 3-1: GSI, HSI AND MICROSOMAL COMPONENTS IN SCUP AND WINTER FLOUNDER

Species(c)	N	GSI(d)	HSI(e)	Cytochrome P-450 nmol/mg	Cytochrome b_5 nmol/mg	NADPH-Cyto. c Reductase nmol/min/mg
Scup, May						
Male, m	6	5.9 ± 1.5	0.50 ± 0.11[b]	0.25 ± 0.09	0.062 ± 0.018	72.9 ± 26.1[a]
Female, m	6	8.6 ± 2.5	0.85 ± 0.19	0.19 ± 0.17	0.054 ± 0.041	43.7 ± 15.7
Scup, June						
Male, m	5	4.1 ± 1.7	1.14 ± 0.17[a]	0.59 ± 0.20[b]	0.132 ± 0.023[b]	159 ± 59
Female, m	6	4.9 ± 1.9	1.51 ± 0.23	0.20 ± 0.10	0.058 ± 0.018	104 ± 15
Winter Flounder, November						
Male, m	5	9.04 ± 2.23	1.11 ± 0.35[b]	1.19 ± 0.18[b]	0.092 ± 0.019[a]	
Female, m	8	9.08 ± 2.26	2.29 ± 0.42	0.42 ± 0.18	0.051 ± 0.028	
Winter Flounder, January						
Male, m	10	7.82 ± 0.99	1.49 ± 0.47[b]	1.28 ± 0.49[b]	0.098 ± 0.029[b]	34.5 ± 17.0[b]
Female, m	5	17.75 ± 3.04	2.67 ± 0.35	0.20 ± 0.11	0.017 ± 0.008	9.1 ± 6.2
Male, i	3	0.14 ± 0.01	1.35 ± 0.38[b]	1.45 ± 0.68	0.144 ± 0.084	37.6 ± 13.5
Female, i	7	0.75 ± 0.17	1.50 ± 0.28[b]	0.83 ± 0.29	0.098 ± 0.030	31.7 ± 24.3

[a,b]Males and females significantly different, [a] $P<0.05$; [b] $P<0.01$
[c]m = gonadally mature; i = gonadally immature.
[d]GSI = gonadosomatic index; (gonad weight/body weight) x 100
[e]HSI = hepatosomatic index; (liver weight/body weight) x 100

flounder of either sex constituted less than 1%/o of the body weight. Local winter flounder breed in winter and early spring, and those with immature gonads were probably adults that had yet to initiate measurable gonadal growth.

The hepatosomatic indices of the mature female scup and winter flounder were slightly (less than 2-fold) but significantly greater than the males'. The livers of the female winter flounder appeared yellowish, as opposed to the red livers in males. The liver of a pre-spawning female fish is often enlarged because vitellogenesis, or yolk protein synthesis, is taking place (van Bohemen et al, 1981; Lindstrom-Seppa, 1985). The livers of these female winter flounder and scup were enlarged and yellowish, presumably due to vitellogenic activity. By comparison, the HSI were not sexually differentiated in immature winter flounder collected at the same time and the livers of the immature females were reddish.

In the winter flounder and in scup collected in June, the specific content of microsomal cytochrome P-450 (nmol/mg protein) was 3 to 6-fold lower in females than in males (Table 3-1). The CO-bound, reduced absorbance maxima were not sexually differentiated in any group (not shown). Shoulders in the absorbance spectra at approximately 420 nm, indicators of cytochrome P-450 degradation to a catalytically inactive form, was not observed. The sex difference in the specific content of cytochrome P-450, then, was not due to differential degradation to cytochrome P-420. The specific content of microsomal cytochrome b_5 was also lower in the female June scup and in the mature female winter flounder than in males. However, the specific contents of cytochromes P-450 and b_5 were not sexually differentiated in scup collected in May, but instead were present at concentrations similar to those in female June scup. Within each sampling of scup or winter flounder, the specific contents of cytochromes P-450 and b_5 in females represented similar proportions of the specific content in males.

NADPH-cytochrome P-450 reductase activities were variable in both teleost species. Reductase activity in male scup collected in May almost 2-fold greater than in females, but was not differentiated in the June scup. In mature female winter flounder from January reductase activity tended to be lower than in mature males or in

immature flounder of either sex. Mean reductase activity was nearly 3-fold lower than gonadally mature female winter flounder than in the mature males.

Ethoxyresorufin O-deethylation: Microsomal EROD activities normalized to protein were significantly lower in female winter flounder and June scup than in males (Table 3-2). The greatest sex difference was observed in mature flounder collected in January, in which mean activity in females was nearly 40 times lower than in males. EROD activity in immature winter flounder was also sexually differentiated, but not the great extent observed in mature fish. In the microsomes from the May scup, EROD activity expressed per mg protein was not sexually differentiated, and was at approximately the same levels as female June scup. EROD activity in the immature scup collected in September was not sexually differentiated, and was as high as the levels observed in mature male June scup.

Dividing monooxygenase activity by cytochrome P-450 content yields the turnover value, indicating the relative levels of spectral cytochrome P-450 metabolizing the substrate. Turnover values for EROD activity were 2-3 times lower in November female winter flounder than in males. In winter flounder collected in January, the mean turnover value was 9-times lower in females than in mature males and immature fish. This suggested that the relative level of P-450 isozymes metabolizing 7-ethoxyresorufin had decreased, and observing the phenomenon in two samples of winter flounder indicated that differentiation was specific to gonadally mature females. Turnover values for EROD activity in June scup were also significantly lower in females than in males, but the difference was only 2-fold. Mean EROD turnover values for May scup were sexually differentiated, but with the opposite trend: statistically, turnover values in females was significantly greater than in males, but the standard deviation was high. In contrast, EROD turnover values for non-reproductive scup collected in September demonstrated no sex differences.

APDM and testosterone 6β-hydroxylation: APDM activity per mg protein and per nmol P-450 was not sexually differentiated in scup (Table 3-3). APDM activity in female January winter flounder was significantly lower than that in males for both mature and immature fish. The decrease in specific APDM activity in winter flounder

TABLE 3-2: MICROSOMAL ETHOXYRESORUFIN O-DEETHYLATION IN SCUP AND WINTER FLOUNDER

Species[c]	N	Ethoxyresorufin O-deethylation nmol/min/mg	nmol/min/nmol
Scup			
May			
Male, m	6	0.58 ± 0.56	1.97 ± 1.41[a]
Female, m	6	0.67 ± 0.21	5.07 ± 2.75
June			
Male, m	5	2.75 ± 1.24[b]	4.66 ± 1.63[b]
Female, m	6	0.46 ± 0.33	2.18 ± 0.74
September			
Male, i	8	3.75 ± 1.92	5.18 ± 2.16
Female, i	4	5.07 ± 4.38	6.39 ± 3.30
Winter Flounder			
November			
Male, m	5	3.680 ± 0.580[b]	3.162 ± 0.756[b]
Female, m	8	0.566 ± 0.442	1.290 ± 0.565
January			
Male, m	10	2.057 ± 0.680[b]	1.757 ± 0.683[b]
Female, m	5	0.054 ± 0.085	0.200 ± 0.213
Male, i	3	2.813 ± 0.360[a]	2.263 ± 1.174
Female, i	7	1.680 ± 0.562	2.124 ± 0.621

[a,b]Males and females significantly different, [a] $P<0.05$; [b] $P<0.01$
[c]m = gonadally mature; i = gonadally immature.

TABLE 3-3: MICROSOMAL APDM AND TESTOSTERONE 6β-HYDROXYLATION IN SCUP AND WINTER FLOUNDER

	N[d]	Aminopyine N-demethylation		Testosterone 6β-hydroxylation	
		unit[c]/mg	unit/nmol	unit/mg	unit/nmol
Scup, May					
M, m	6	0.91 ± 0.39	3.75 ± 1.79	0.016 ± 0.018	0.054 ± 0.049
F, m	6	0.55 ± 0.46	3.13 ± 1.10	0.012 ± 0.014	0.060 ± 0.028
Winter Flounder, January					
M, m	10	1.66 ± 0.40[b]	1.32 ± 0.50[b]	0.044 ± 0.016	0.037 ± 0.015[b]
F, m	5	0.94 ± 0.19	5.46 ± 1.56	0.027 ± 0.018	0.173 ± 0.142
M, i	2	2.58 ± 0.28[b]	2.22 ± 1.59	0.025 ± 0.011	0.023 ± 0.020
F, i	3	1.44 ± 0.16	2.35 ± 0.94	0.037 ± 0.014	0.052 ± 0.022

M = male; F = female; m = gonadally mature; i = gonadally immature.
[a,b]Males and females significantly different, [a] $P<0.05$; [b] $P<0.01$
[c]units = nmol product (HCHO or 6β-OH-testosterone) formed per minute
[d]Testosterone metabolism: immature males, N = 3; immature females, N = 4

was small relative to the 40-fold sex difference observed for EROD activity. The APDM turnover values were greater in mature female winter flounder than in males, and were not sexually differentiated in immature fish. This suggested that in mature winter flounder the microsomal P-450 population in females contained a greater abundance of catalysts(s) of AP demethylation, and fewer catalysts of EROD activity, than did microsomes from males.

The range of microsomal testosterone 6β-hydroxylase activity in gonadally mature May scup was 0.003 to 0.051 nmol/min/mg, and 0.022 to 0.151 nmol/min/nmol P-450. These ranges encompass the reported mean values for microsomal testosterone 6β-hydroxylation in both gonadally mature winter flounder and brook trout (Stegeman and Woodin, 1984). Testosterone 6β-hydroxylation normalized either to protein or to cytochrome P-450 was not sexually differentiated in the May scup (Table 3-3). Activity per mg protein was also not sexually differentiated in January winter flounder. This contrasted with the patterns of EROD and APDM activities, which, with E_2 2-hydroxylation (discussed below), were lower in microsomes from mature females than from other groups of winter flounder. The turnover values for testosterone 6β-hydroxylation were variable in mature female winter flounder, but were greater in mature females than in mature males. Turnover values were not sexually differentiated between immature fish. This sex difference parallels that of APDM activity in winter flounder, and both were opposite from the patterns of sexual differentiation of EROD activity in winter flounder (Table 3-2).

Estradiol 2-hydroxylation: The analysis of microsomal E_2 hydroxylase activity in scup and winter flounder was presented in chapter 2. Additional analyses of scup collected in May and of gonadally immature male winter flounder are presented here and compared with previous results. Specific E_2 2-hydroxylase activities were similar between June scup and winter flounder microsomes, and were approximately ten times greater than activity catalyzed in liver microsomes from scup collected in May (Table 3-4). As already shown (Table 2-3), the rate of microsomal E_2 2-hydroxylation per mg protein was significantly greater in gonadally mature males than females of both species. In winter flounder, specific activity was not sexually differentiated between immature

TABLE 3-4: MICROSOMAL ESTRADIOL 2-HYDROXYLATION IN SCUP AND WINTER FLOUNDER

Species[c]	N	Estradiol 2-Hydroxylation nmol/min/mg	nmol/min/nmol
Scup			
May			
Male, m	6	0.038 ± 0.018[a]	0.152 ± 0.069
Female, m	6	0.017 ± 0.012	0.103 ± 0.073
June			
Male, m	5	0.362 ± 0.141[b]	0.608 ± 0.142
Female, m	6	0.163 ± 0.048	0.889 ± 0.224
Winter Flounder			
November			
Male, m	5	0.332 ± 0.056[b]	0.284 ± 0.064
Female, m	8	0.100 ± 0.013	0.257 ± 0.093
January			
Male , m	8	0.274 ± 0.087[b]	0.222 ± 0.109[a]
Female, m	5	0.076 ± 0.025	0.481 ± 0.239
Male, i	3	0.299 ± 0.027	0.235 ± 0.099
Female, i	6	0.224 ± 0.083	0.257 ± 0.058

[a,b]Males and females significantly different, [a] $P<0.05$; [b] $P<0.01$
[c]m = gonadally mature; i = gonadally immature.
Some of this data was presented in Snowberger and Stegeman, 1987, and are shown in table 2-3.

animals. In microsomes from mature January winter flounder, the turnover values for E_2 2-hydroxylation were slightly but significantly greater in females than in males. However, the turnover values were not sexually differentiated in the scup or in the November winter flounder. The turnover values for the May scup were lower than for the June scup or the range of activity observed in gonadally immature scup (0.5 to 1.0 nmol/min/nmol; Klotz et al, 1986).

Composition of Scup and Winter Flounder Microsomes

Vitellogenin: It was of interest to determine if vitellogenin was present in hepatic microsomes from pre-spawning female teleosts, as it was possible that it could contribute toward decreasing calculated microsomal parameters normalized to protein. To examine this possibility, microsomal proteins from killifish were separated on 7.5%/o SDS-PAGE gels and immunoblotted against anti-killifish vitellogenin. A 200 kD protein, of the same molecular weight as vitellogenin (Selman and Wallace, 1983), was detected in hepatic microsomes and serum from gravid females (Figure 3-1). Lower molecular weight immunoreactive proteins, which may have been degradation products, were also present in serum. This indicated for the first time that vitellogenin was indeed present in hepatic microsomes from gravid female fish. It is unknown, however, if vitellogenin is present at levels that could significantly decrease monooxygenase activities normalized to microsomal protein.

Anti-P-450E as probe: Both AHH and EROD activities in winter flounder microsomes were inhibited by polyclonal antibodies against scup P-450E (Stegeman, Woodin, and Gray, unpub. obs.). Both polyclonal anti-P-450E and MAb 1-12-3 against P-450E recognized a distinct protein, about 55-56 kD, in immunoblotted winter flounder microsomes. In addition, BNF treatment of gonadally regressed winter flounder induced the levels of this protein with concomitant increases in EROD and AHH activities (Stegeman et al, 1987). Therefore, it is apparent that MAb 1-12-3 recognizes a winter flounder homolog of scup P-450E in immunoblots.

The levels of cytochrome P-450E and its winter flounder homolog were quantified by immunoblotting microsomes from scup and winter flounder against MAb 1-12-3. Only microsomes from animals collected from the same site at the same time were compared for sex differences,

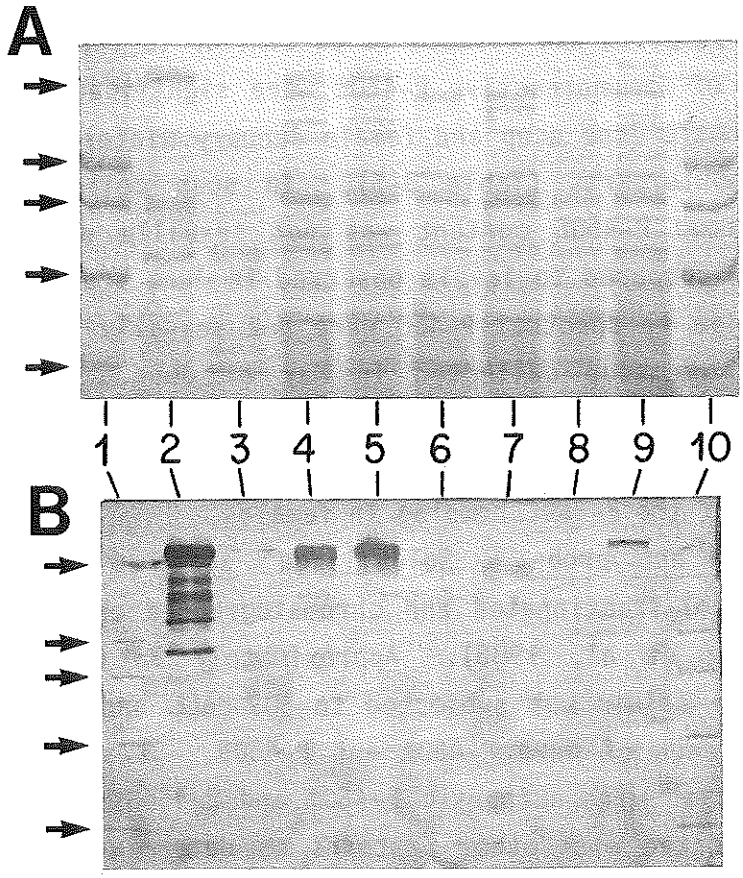


Figure 3-1. Anti-killifish vitellogenin immunoblotted against sera and hepatic microsomes from killifish. A. Ponceau-stained nitrocellulose following transfer of electrophoresed proteins from SDS-PAGE gels. B. Same nitrocellulose after immunoblotting against anti-vitellogenin. Lane 1) Arrows mark molecular weight standards of (from top) 200, 116.25, 97.4, 66.2, and 42.7 kD; 2) 0.3 µl plasma from a gravid female; 3) 0.3 µl plasma from a ripe male; 4 and 5) 50 µg microsomal protein from 2 gravid females; 6 and 7) 50 µg microsomal protein from 2 ripe males; 8 and 9) 50 µg microsomal protein from 2 regressed females; 10) molecular weight standards as in Lane 1.

to minimize variations due to induction of P-450E by environmental chemicals (Kloepper-Sams et al, 1987; Stegeman et al, 1987). The affinity of MAb 1-12-3 for the winter flounder homolog is unknown, but for calculation purposes was assumed to be similar to that for P-450E.

The levels of immunoreactive P-450E were significantly lower per µg protein in female June scup and female January winter flounder than in the male counterparts (Table 3-5). In scup collected in May, specific cytochrome P-450E content was not sexually differentiated, and appeared low compared to levels observed in June scup. The levels of P-450E relative to spectral P-450 were not sexually differentiated in microsomes from scup collected in May or June. The level of microsomal P-450E relative to spectral P-450 was significantly lower in female winter flounder than in males, possibly indicating down-regulation of the P-450E homolog in females.

Anti-P-450A as probe: Solubilized scup microsomes were polyethylene glycol-fractionated and chromatographed on DE-52 columns as before (Klotz et al, 1986). Approximately 33 mg of the A/B fraction from the column was subjected to SDS-PAGE and electroelution to yield 700 µg each of pure P-450A and P-450B. These electroeluted proteins co-migrated with authentic P-450A and P-450B on SDS-PAGE and were not contaminated with other proteins as determined by Coomassie blue staining. Immunoblots demonstrated no cross-reactivity of either protein with MAb 1-12-3 (anti-P-450E) or anti-trout LM2 (not shown). Anti-trout LM2 is a polyclonal antibody to trout LM-2 (see Table 1-4) that recognized a protein in scup P-450 fractions C and D, but not previously purified P-450A or P-450B, in immunoblots (unpub. obs.). The silver-stained peptide maps of P-450A, P-450B, and the electroeluted proteins showed many bands, yet examination of the gels demonstrated distinct fragments common only to the standards and their counterparts (Figure 3-2a, b). In conjunction with their elution profile from DE-52 columns and co-migration with standard on SDS-PAGE, the electroeluted proteins were assumed to be P-450A and P-450B.

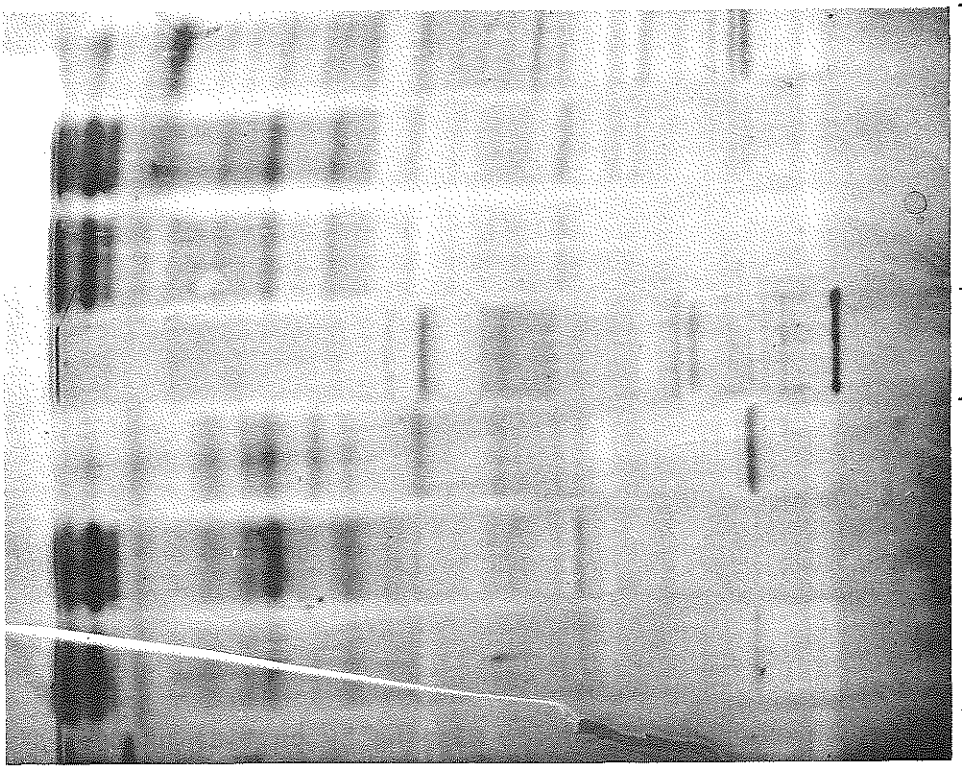
Polyclonal antibodies against electroeluted P-450A and P-450B were tested in immunoblots against various purified and partially purified scup P-450s. Anti-P-450A recognized both electroeluted and previously purified scup P-450A, and proteins in P-450 fractions C and D that comigrated with P-450A, but did not recognize P-450B or P-450E

TABLE 3-5: MICROSOMAL P-450E AND P-450A CONTENT IN MATURE SCUP AND WINTER FLOUNDER

Species[c]	N	Microsomal P-450E		Microsomal P-450A	
		nmol/mg	nmol/nmol	nmol/mg	nmol/nmol
Scup					
May					
Male	4	0.04 ± 0.03	0.13 ± 0.04	0.08 ± 0.02	0.34 ± 0.13
Female	4	0.02 ± 0.01	0.10 ± 0.03	0.05 ± 0.02	0.38 ± 0.22
June					
Male	4	0.22 ± 0.06[b]	0.42 ± 0.10	0.21 ± 0.10	0.36 ± 0.08
Female	6	0.07 ± 0.06	0.32 ± 0.14	0.11 ± 0.06	0.63 ± 0.30
Winter Flounder, January[d]					
Male	10	0.53 ± 0.21[b]	0.43 ± 0.13[b]	0.26 ± 0.10	0.21 ± 0.08[b]
Female	5	0.02 ± 0.02	0.13 ± 0.10	0.20 ± 0.03	1.22 ± 0.48

[a,b]Significantly different from females, [a] $P<0.05$ or [b] $P<0.01$ by student's t-test or Mann-Whitney U test.
[c]All animals examined were gonadally mature.
[d]Units are scup isozyme equivalents per mg microsomal protein or per nmol P-450.

Figure 3-2a. Cytochrome P-450A peptide mapping by partial proteolysis with α-chymotrypsin and Staphylococcus aureus V8 protease. Digestions of electroeluted P-450A (eP-450A) or previously purified P-450A were performed in 0.25°/o SDS for 90 min at 37°C as described in the methods section. The digests were analyzed by discontinuous SDS-PAGE on a 13.5°/o separating gel. Protein samples (5 μg) were loaded in each lane after incubation with (from left to right) with 0, 1, and 5 μg α-chymotrypsin per ml or 0, 5, and 20 μg V8 protease per ml. MW refers to molecular weight standards.


eP-450A
MW
P-450A
CT

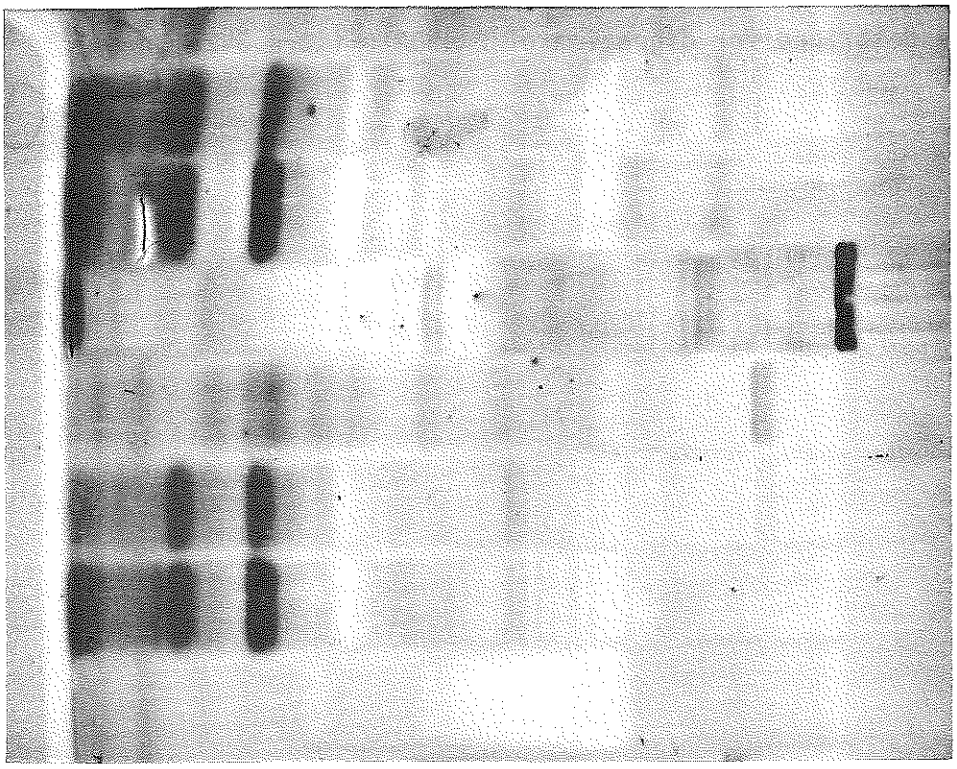
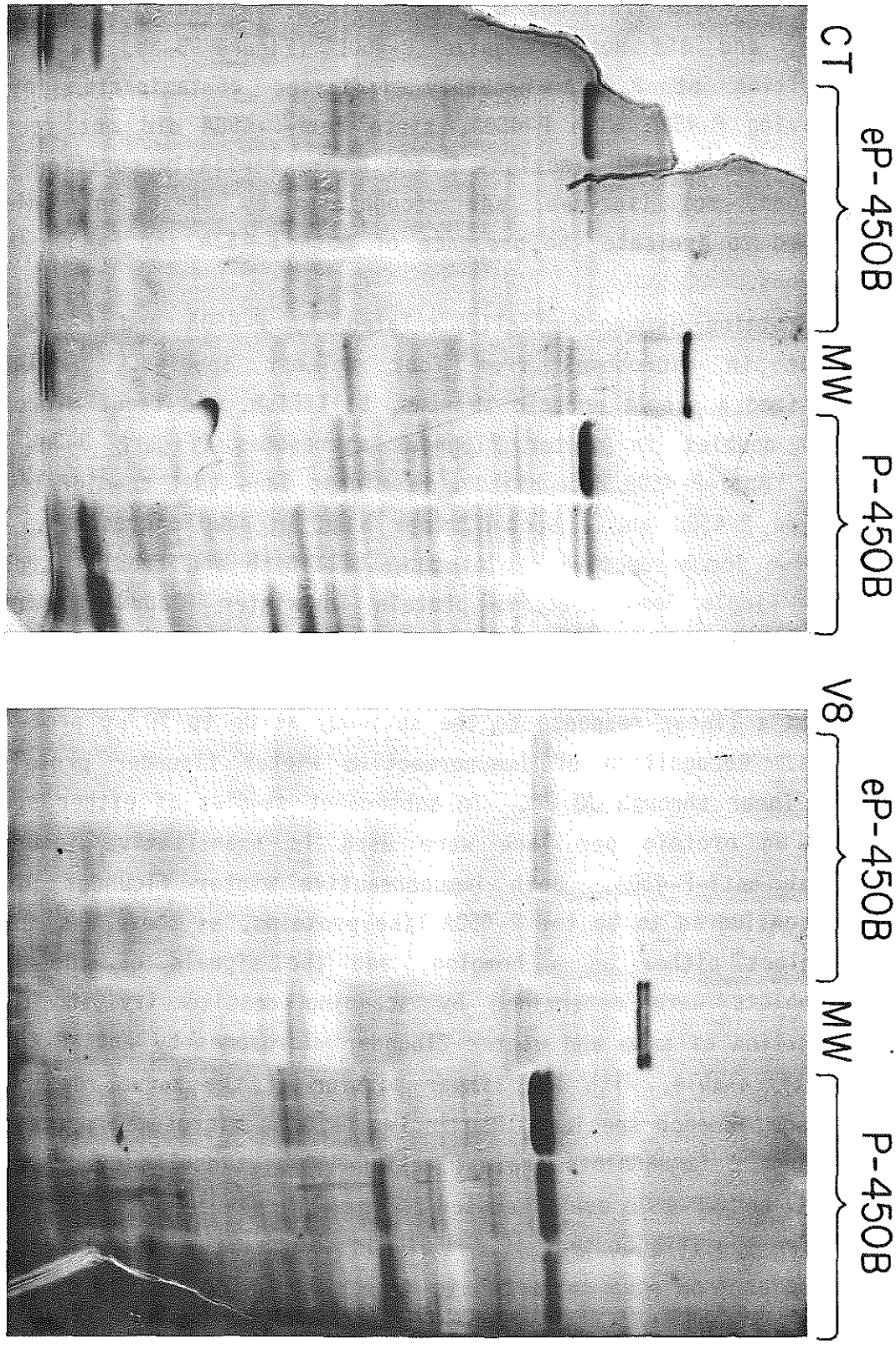

P-450A
MW
eP-450A
V8

Figure 3-2b. Cytochrome P-450B peptide mapping by partial proteolysis with α-chymotrypsin and Staphylococcus aureus V8 protease. Digestions of electroeluted P-450B (eP-450B) or previously purified P-450B were performed as described for digestion of P-450A (Figure 3-2a).


CT
eP-450B
MW
P-450B
V8
eP-450B
MW
P-450B

(Figure 3-3a, lanes 1-7). Anti-P-450B recognized both electroeluted and previously purified P-450B and proteins of similar molecular weight in P-450 fractions C and D. It cross-reacted somewhat with P-450A, and did not recognize P-450E (Figure 3-3b, lanes 1-7). Recognition of P-450A probably indicated contamination of the immunizing P-450B with P-450A, since anti-P-450A did not recognize P-450B. Both P-450 fractions C and D are partially purified column fractions, not isozymes, and recognition by these antibodies is believed to indicate the presence of both P-450A and P-450B in the fractions.

Proteins immunochemically related to P-450A and P-450B were present in microsomes from four teleost species. Anti-P-450A recognized a single protein in scup, killifish, and trout microsomes, and a doublet in winter flounder microsomes (Figure 3-3a, lanes 8-11). Anti-P-450B recognized a prominent band that co-migrated with purified P-450B and a second minor band in scup microsomes. There were two immunoreactive proteins in killifish and trout microsomes, and a single immunoreactive protein in winter flounder microsomes (Figure 3-3b, lanes 8-11).

Titration of scup microsomes against anti-P-450A in immunoblots yielded a linear response to the antibody at up to 70 μg protein per sample. Recognition of immunoreactive winter flounder protein was also linear through 70 μg. In subsequent studies of either species, 20-50 ug protein per lane were used in quantitative immunoblots against anti-P-450A. Both immunoreactive winter flounder proteins were considered to be the P-450A-like proteins, as there was no basis to select either as a homolog, and the signals of the two on immunoblots were determined by scanning densitometry and summed. Recognition of scup and winter flounder microsomes by anti-P-450B was also titratable, with the linear portion of the curve ranging from 5-20 μg for each species. Routinely, 5-20 μg microsomal protein were employed in immunoblots against anti-P-450B. (Results of immunoblots against anti-P-450B are discussed in the Appendix.)

The specific content of P-450A was not sexually differentiated in microsomes from scup collected in May or June, and in general appeared to be slightly lower in the former group than in the latter (Table 3-5). The levels of P-450A relative to total spectral P-450 were also

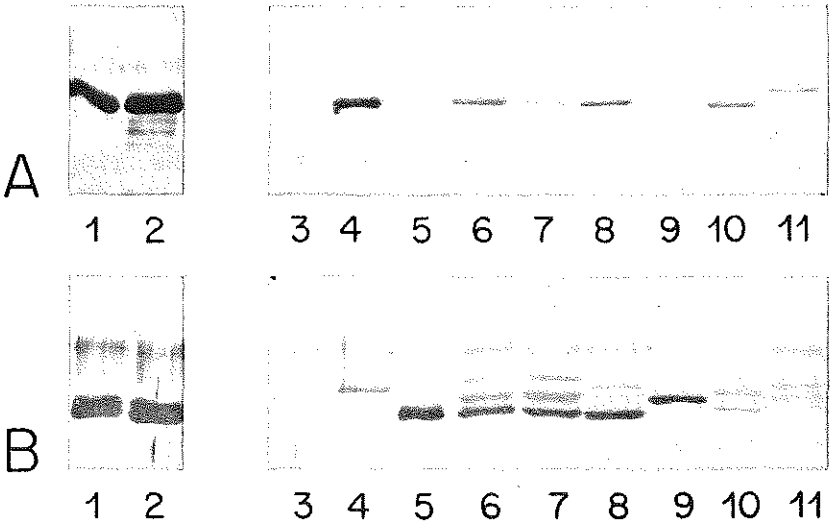


Figure 3-3. Anti-P-450A and anti-P-450B immunoblotted against scup isozymes and teleost microsomes. Each lane contained 20 pmol of cytochrome P-450. A. Crude anti-P-450A antiserum immunoblotted against 1) electroeluted P-450A and 2) previously purified P-450A; purified anti-P-450A IgG blotted against 3) scup P-450E, 4) eluted scup P-450A, 5) eluted scup P-450B, 6) scup P-450 fraction C, 7) scup P-450 fraction D, microsomes from 8) scup, 9) winter flounder, 10) killifish, and 11) rainbow trout. B. Crude anti-P-450B antiserum immunoblotted against 1) eluted P-450B and 2) previously purified P-450B. Lanes 3-11: Contents of each lane as in part A, immunoblotted against purified anti-P-450B IgG.

not sexually differentiated in the gonadally mature scup, and appeared comparable between the two groups. The specific content of the anti-P-450A reactive protein in winter flounder was not different between mature male and female winter flounder. Yet, P-450A levels relative to total cytochrome P-450 were 5-6 times greater in female winter flounder than in males. These sex differences in the composition of the P-450 population will be considered in discussing the mechanisms of sex differences in teleost monooxygenase activities.

Correlations

Examination of microsomes with anti-P-450A was conducted exclusively by immunoblotting. Preincubation with anti-P-450A did not inhibit testosterone 6β-hydroxylation in scup microsomes, although 6β-OH-testosterone was previously shown to be the single product of testosterone metabolism by reconstituted P-450A (Klotz et al, 1986). Reconstituted P-450A also catalyzed E_2 2-hydroxylation, yet the antibody had no effect on this activity either. Exposing the P-450A epitopes by solubilizing microsomes in 0.4%/o cholate and 0.2%/o Emulgen as for purification did not render either activity susceptible to inhibition by anti-P-450A. The possibility that anti-P-450A only recognized denatured proteins, such as those in immunoblots, was tested with Ouchterlony double-diffusion assays. Solubilized microsomal proteins with no cytochrome P-420 (the presence of P-420 would have indicated degradation) were recognized by anti-P-450A in double-diffusion assays, indicating some affinity of the antibody for native proteins despite its inability to inhibit microsomal activity. Therefore, anti-P-450A did recognize epitopes in the microsomes, but its inability to inhibit activities mediated by reconstituted P-450A suggested that it did not bind sites strategic for inhibiting catalysis. Therefore, the contributions of P-450A to microsomal monooxygenase activities was inferred through correlation analyses.

If an isozyme catalyzes an activity, or if two activities are catalyzed by the same isozyme, the relationship may be indicated by a positive correlation between isozyme level and enzyme activity, or between the two enzyme activities, respectively. Correlations were calculated between isozyme levels and monooxygenase activities in winter flounder and scup microsomes to detect these relationships. Several positive correlations were determined between parameters

normalized to microsomal protein. Correlation coefficients between parameters normalized to cytochrome P-450 were also determined in case parameters per unit protein were correlated through general suppression of those values in mature females. In association with background information about P-450 isozymes and monooxygenase activities, positive correlations between parameters normalized to both microsomal protein and cytochrome P-450 were considered to most strongly indicate biologically significant relationships.

Scup: Data from the May and June scup were combined into one dataset from which to calculate correlation coefficients. There were several significant, positive correlations between monooxygenase activities per mg protein and the specific content of P-450 isozymes (Table 3-6, top). P-450E content was positively correlated with EROD, APDM, E_2 2-hydroxylase, and testosterone 6β-hydroxylase activities. The specific content of P-450A was also positively correlated with specific EROD and E_2 2-hydroxylase activities. Positive correlations indicate that levels of activity co-varied with isozyme content, but do not reveal if the relationship is causative. For example, the positive correlation between specific EROD activity and the specific content of P-450E reflects the known causal relationship between the two (Kloepper-Sams et al, 1987). However, the specific contents of microsomal P-450E and P-450A were positively correlated with each other, yet it is unlikely that the levels of P-450E directly influenced the levels of P-450A or vice versa. To reiterate, correlation indicates a mathematical relationship between parameters, but background knowledge is necessary to determine if a biologically significant relationship exists between them.

In contrast to the correlation of data per mg protein, EROD turnover values and P-450E per nmol P-450 were not correlated with each other in scup (Table 3-6, bottom). This was especially apparent in mature females collected in May, in which EROD turnover values were higher than in males, yet P-450E per nmol P-450 was not sexually differentiated. Because P-450E is the apparent major catalyst of microsomal EROD activity, and P-450E levels correlated with scup EROD activity in other studies (Kloepper-Sams, pers. comm.), the lack of correlation with EROD activity in these samples was unexpected. Several factors might have contributed to this paradox. To quantify

TABLE 3-6: CORRELATION COEFFICIENTS BETWEEN MICROSOMAL P-450 ISOZYME CONTENT AND MONOOXYGENASE ACTIVITIES IN SCUP

	EROD/mg	APDM/mg	E_2 2-OH/mg	T 6β-OH/mg	P-450E/mg
APDM/mg[d]	0.258				
E_2 2-OH/mg	0.765[c]	0.877[c]			
T 6β-OH/mg	0.664[a]	0.644[a]	0.661[a]		
P-450E/mg[e]	0.840[c]	0.616[a]	0.873[c]	0.689[a]	
P-450A/mg	0.733[c]	0.408	0.865[c]	0.381	0.691[c]

	EROD TO	APDM TO	E_2 2-OH TO	T 6β-OH TO	P-450E/nmol
APDM TO[d]	-0.088				
E_2 2-OH TO	-0.158	0.801[b]			
T 6β-OH TO	0.229	0.002	0.312		
P-450E/nmol[e]	0.156	0.061	0.618[b]	-0.075	
P-450A/nmol	0.019	0.031	0.602[b]	0.031	0.020

[a-c]Correlation coefficient significance: [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$
[d]Monooxygenase activities per minute expressed per mg microsomal protein or as turnover values (TO).
[e]P-450E and P-450A nmol/mg microsomal protein or nmol/nmol P-450.
There were 11-22 degrees of freedom for each comparison.

specific isozyme content, 10-20 µg microsomal protein were immunoblotted against anti-P-450E (MAb 1-12-3). The level of P-450E per nmol P-450 was then determined by dividing the specific content of P-450E by the specific content of cytochrome P-450. If the specific content of cytochrome P-450 was low, the calculated value of P-450E per nmol P-450 might be overestimated, distorting the known association between P-450E content and EROD activity. To illustrate, if only those scup are compared for which microsomal P-450 content was greater than or equal to 0.15 nmol/mg, the correlation between EROD turnover values and P-450E per nmol P-450 became significant ($N = 16$, $r = 0.63$, $P<0.01$).

In addition, the availability of microsomal NADPH-cytochrome P-450 reductase to the cytochrome P-450 could have affected EROD activity. In rats, microsomal activity appeared to be reductase-limited in some circumstances. Exogenous reductase slightly stimulated cytochrome P-450 mediated reduction of amarinth by microsomes from phenobarbitone-treated but not control rats (Mallett et al, 1985), and modestly stimulated warfarin metabolism by microsomes from rats treated with various inducers (Kaminsky and Guengerich, 1985). A preliminary study indicated that microsomal E_2 2-hydroxylase activity was not increased in tetrachlorodibenzo-p-dioxin treated male rats assayed in the absence of added reductase, but was increased 2-fold in the presence of exogenous reductase (Graham et al, 1987). Impaired P-450-reductase interactions could have similarly affected EROD activities and thus contributed toward the lack of correlation between P-450E content and EROD activity in the scup samples. However, exogenous reductase was not added to assays of winter flounder and scup microsomes as it had not stimulated previous assays of microsomal activity in immature fish (Stegeman and Woodin, unpub. obs.). Furthermore, the ratio of reductase activity to P-450 content was not reduced in the gonadally mature scup and winter flounder. It might be speculated that proteins or lipids unique to microsomes from spawning fish hindered P-450-reductase interactions causing underestimation of monooxygenase activities.

P-450E content per nmol P-450 was positively correlated with E_2 2-hydroxylase turnover values in scup (Table 3-6), although it does not appear to be an E_2 2-hydroxylase (Snowberger and Stegeman,

1987). Therefore, the positive correlation indicates co-variance, not a causal relationship. P-450A levels and E_2 2-hydroxylase activities were also positively correlated in scup, both per mg protien and per nmol P-450, shown in regression curves in Figure 3-4. Reconstituted P-450A catalyzed E_2 2-hydroxylation (Klotz et al, 1986), and the positive correlation here might reflect activity by microsomal P-450A. P-450A levels were not correlated with microsomal testosterone 6β-hydroxylase activity, although it was catalyzed by reconstituted P-450A (Klotz et al, 1986). The lack of a correlation between microsomal P-450A and testosterone 6β-hydroxylation could indicate that P-450A does not contribute significantly to microsomal activity, and would require further analysis to resolve.

Of the four monooxygenase activities, only APDM and E_2 2-hydroxylase activities were positively correlated with each other whether normalized to protein or to cytochrome P-450. This consistent correlation could indicate AP and E_2 metabolism by co-varying catalysts, or even by the same catalyst. As will be discussed, this correlation was also observed in winter flounder microsomes.

Winter flounder: In winter flounder, the specific content of P-450E was positively correlated with specific EROD activity. The levels of the P-450E homolog per nmol P-450 were also positively correlated with EROD turnover values. This reflects the strong evidence that the winter flounder P-450E homolog is a major catalyst of activity in winter flounder microsomes (Stegeman et al, 1987). The regression curve of P-450E and EROD activity is shown in Figure 3-5. Although regression was significant, the correlation coefficients were less than those in a previous similar study with winter flounder, for which $r > 0.95$ (Stegeman et al, 1987).

The specific content of P-450E was also positively correlated with specific APDM and E_2 2-hydroxylase activities. However, P-450E per nmol P-450 was negatively correlated with turnover values for all monooxygenase activities other than EROD (Table 3-7). The negative correlations indicated that the P-450E homolog did not catalyze these activities, which was expected as anti-P-450E did not inhibit the activities in scup microsomes (Kloepper-Sams et al, 1987; Snowberger and Stegeman, 1987). To summarize the correlation analysis of the P-450E homolog, the specific content of P-450E co-varied with

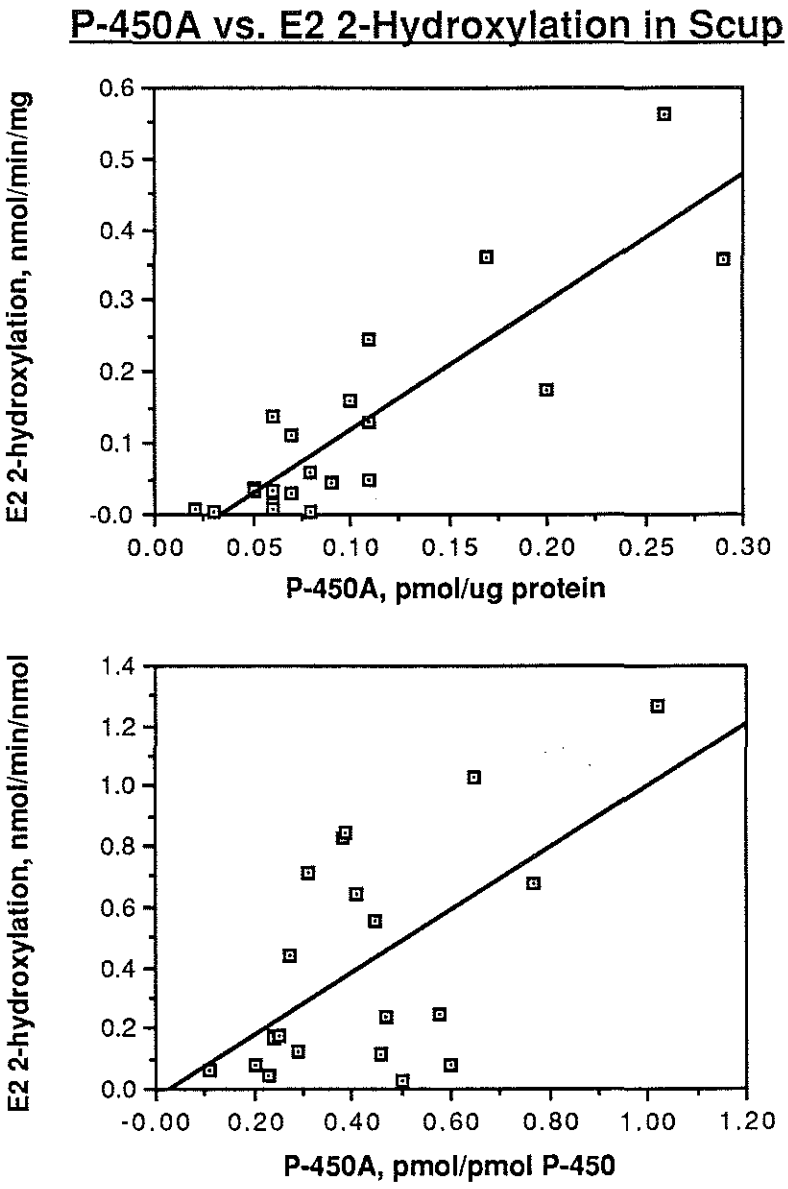


Figure 3-4. Regression between the relative amount of P-450A detected by anti-P-450A and microsomal E_2 2-hydroxylase activity in gonadally mature scup. 20 or 80 µg microsomal protein was applied to each lane of gel. Values on the x-axis are the ratio of P-450A equivalents per µg protein or per pmol spectral cytochrome P-450. N = 21. Above, specific activity: r = 0.865; P<0.001. Below, turnover values: r = 0.602; P<0.01.

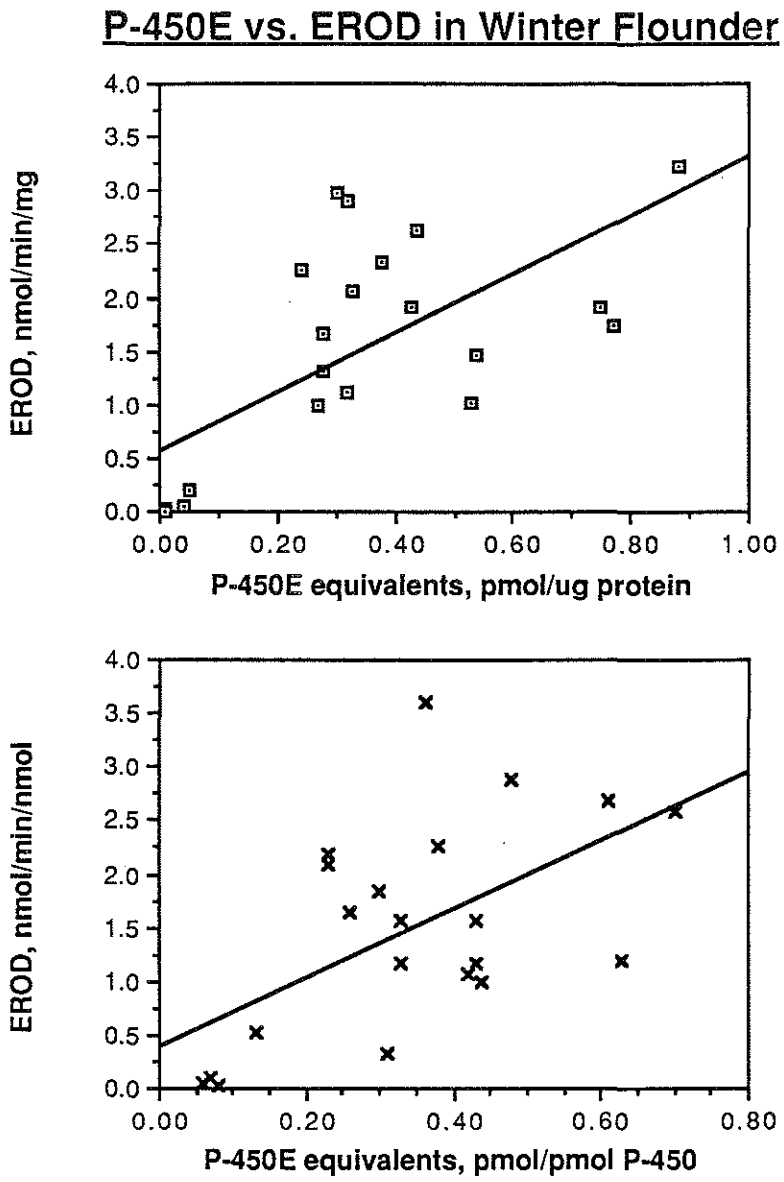


Figure 3-5. Regression between relative amounts of P-450E detected by MAb 1-12-3 and microsomal EROD activity in winter flounder collected in January. 8 or 20 μg microsomal protein was applied to each lane of gel. Values on the x-axis are the ratio of P-450E equivalents per μg protein or per pmol spectral cytochrome P-450. N = 21. Above, specific activity: $r = 0.658$; $P<0.01$. Below, turnover values: $r = 0.571$; $P< 0.01$.

TABLE 3-7: CORRELATION COEFFICIENTS BETWEEN MICROSOMAL P-450 ISOZYME CONTENT AND MONOOXYGENASE ACTIVITIES IN JANUARY WINTER FLOUNDER

	EROD/mg	APDM/mg	E_2 2-OH/mg	T 6β-OH/mg	P-450E/mg
APDM/mg[d]	0.823[c]				
E_2 2-OH/mg	0.780[c]	0.741[c]			
T 6β-OH/mg	0.303	0.222	0.124		
P-450E/mg[e]	0.658[b]	0.608[a]	0.679[b]	0.302	
P-450A/mg	0.207	0.316	0.296	0.569[a]	0.530

	EROD TO	APDM TO	E_2 2-OH TO	T 6β-OH TO	P-450E/nmol
APDM TO[d]	-0.464				
E_2 2-OH TO	-0.341	0.751[c]			
T 6β-OH TO	-0.462[a]	0.715[c]	0.834[c]		
P-450E/nmol[e]	0.571[b]	-0.533[a]	-0.488[a]	-0.546[a]	
P-450A/nmol	-0.656[a]	0.940[c]	0.810[c]	0.780[b]	-0.698[b]

[a-c]Correlation coefficient significance: [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$
[d]Monooxygenase activities per minute expressed per mg microsomal protein or as turnover values (TO).
[e]P-450E and P-450A equivalents per mg microsomal protein or per nmol P-450.
There were 12-20 degrees of freedom for each comparison.

several enzymatic activities relative to microsomal protein in winter flounder. Yet, the specific content of spectral P-450 varied between individuals such that lower levels of P-450E per nmol P-450 were present in microsomes catalyzing APDM, E_2 2-hydroxylase, and testosterone 6β-hydroxylase activities per nmol P-450 at higher rates, presumably with higher catalyst levels in the microsomal P-450 pool.

Results of correlation analysis of P-450E in winter flounder were consistent with predictions, suggesting that similar analysis of P-450A could indicate monooxygenase activities that co-varied with this homolog. The specific content of the P-450A homolog was positively correlated only with specific testosterone 6β-hydroxylase activity (Table 3-7, top). There was also a significant correlation between the P-450A homolog per nmol P-450 and testosterone 6β-hydroxylase turnover values; both regression curves are shown in Figure 3-6. Reconstituted scup P-450A catalyzed this activity (Klotz et al, 1986), although as discussed correlation analyses suggested that the contribution of P-450A to microsomal testosterone 6β-hydroxylation might be limited in the scup examined. In the winter flounder microsomes, however, the correlations suggest that the P-450A homolog could contribute toward microsomal testosterone 6β-hydroxylation. It might be worthwhile to analyze another set of scup microsomes to determine if the lack of correlation in the sample used here actually represents the scup population at large.

There was a positive correlation between APDM turnover values and P-450A levels expressed per pmol P-450, but not when expressed per mg protein. The regression curve is shown in Figure 3-7. It appears that in these winter flounder microsomes, the APDM catalyst(s) co-varied with the P-450A homolog within the spectral P-450 pool but not per unit microsomal protein. The correlation between E_2 2-hydroxylation and P-450A homolog levels per nmol P-450 was also significant, and regression analysis again demonstrated a positive slope (Figure 3-8). As with APDM activity, specific P-450A homolog levels and specific E_2 2-hydroxylase activity were not correlated.

The correlation between P-450A and P-450E levels per pmol spectral cytochrome P-450 was negative in winter flounder, indicating an inverse relationship between the proportions of these isozymes in the microsomal P-450 pool (Table 3-7). Specific EROD, APDM, and E_2

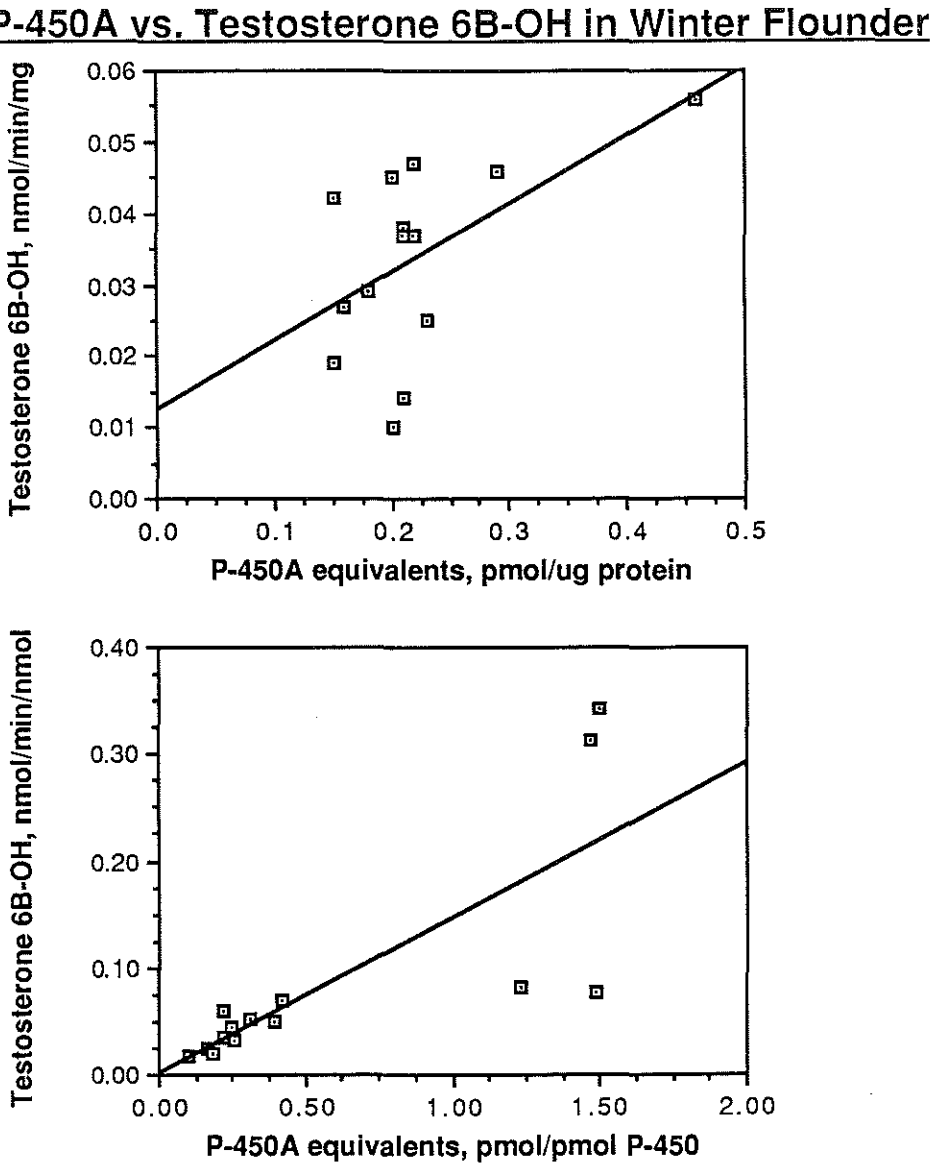


Figure 3-6. Regression between the relative amount of P-450A equivalents detected by anti-P-450A and microsomal testosterone 6β-hydroxylase activity in winter flounder collected in January. 15-20 μg microsomal protein was applied to each lane of gel. Values on the x-axis are the ratio of P-450A equivalents per μg protein or per pmol spectral P-450. N = 14. Above, specific activity: $r = 0.569$; $P<0.05$. Below, turnover values: $r = 0.780$; $P<0.01$

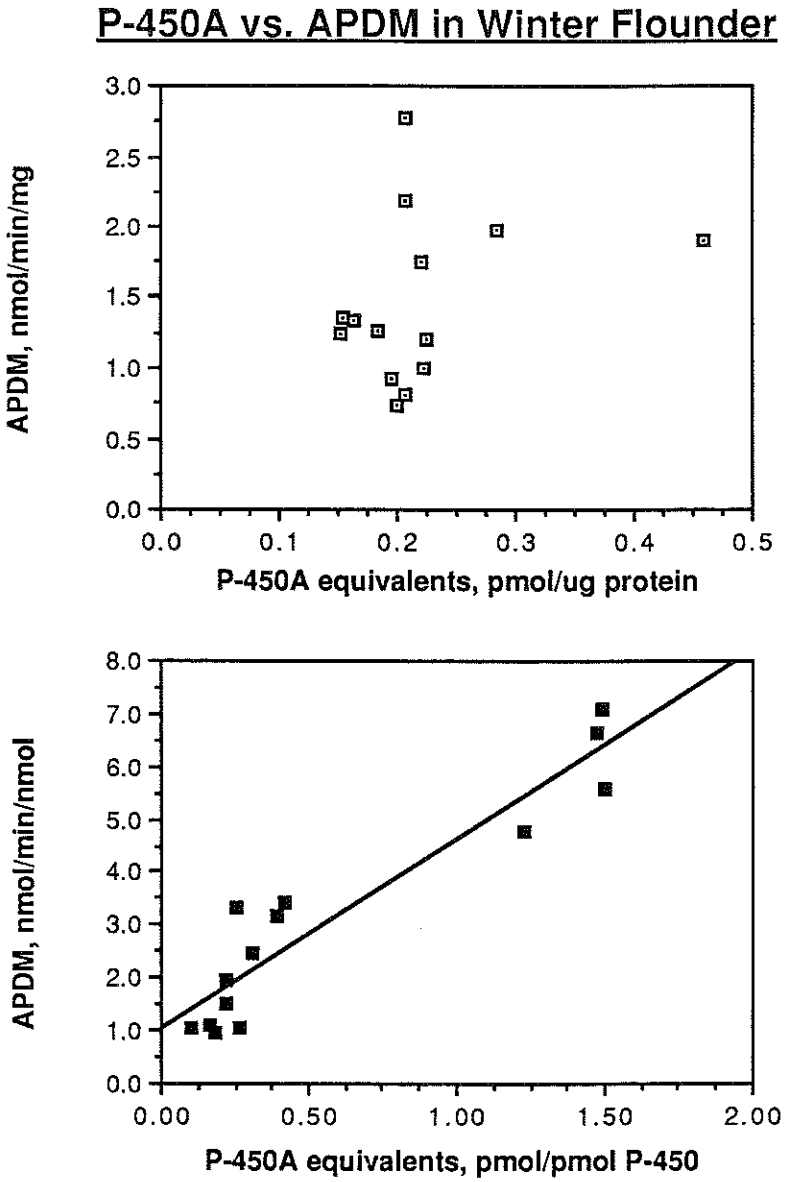


Figure 3-7. Regression between the relative amount of P-450A equivalents detected by anti-P-450A and microsomal APDM activity winter flounder collected in January. 15-20 μg microsomal protein was applied to each lane of gel. Values on the x-axis are the ratio of P-450A equivalents per μg protein or per pmol spectral cytochrome P-450. N = 14. Above, specific activity: r = 0.316, not significant. Below, turnover values: r = 0.940; P<0.001.

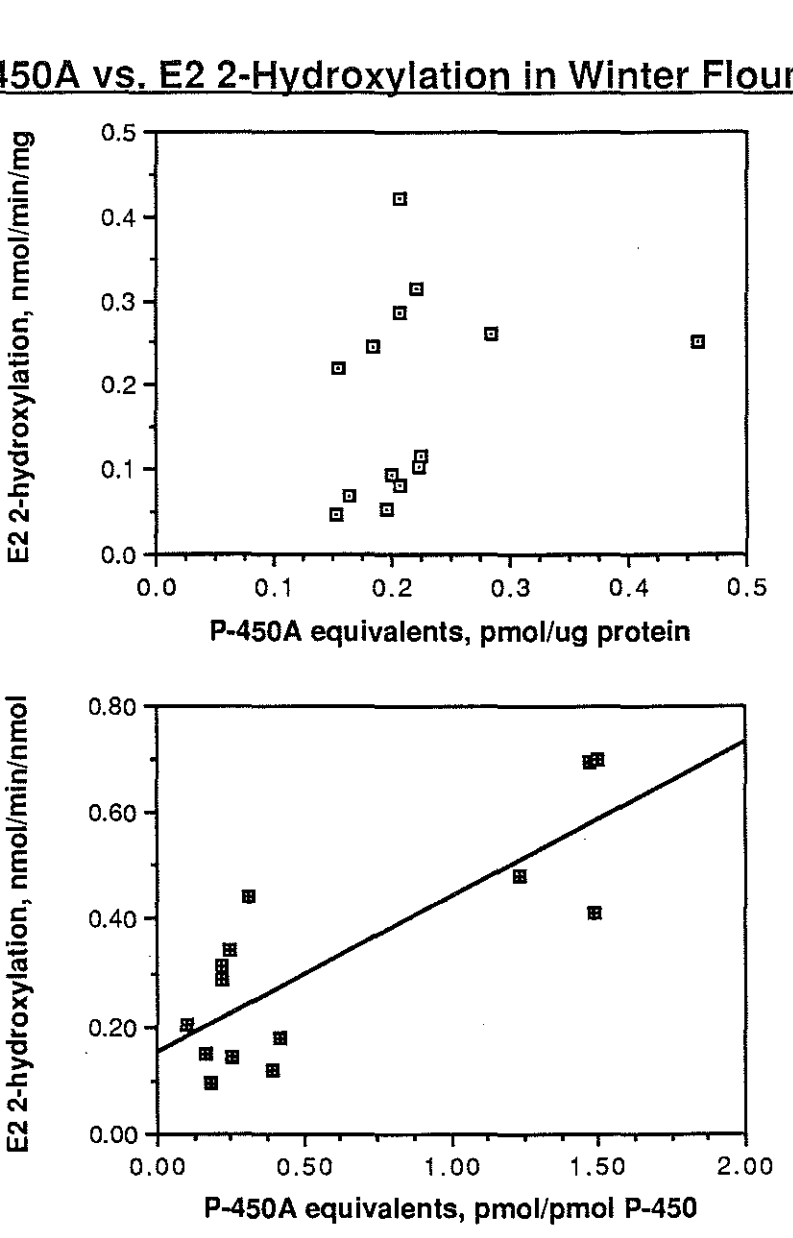


Figure 3-8. Regression between the relative amount of P-450A equivalents detected by anti-P-450A and microsomal E_2 2-hydroxylase activity in winter flounder collected in January. 15-20 µg microsomal protein was applied to each lane of gel. Values on the x-axis are the ratio of P-450A equivalents per µg protein or per pmol spectral cytochrome P-450. N = 14. Avove, specific activity: r = 0.296; not significant. Below, turnover values: r = 0.810; P<0.001.

2-hydroxylase activities were positively correlated with one another, in agreement with all three being lower in mature female winter flounder than in mature males (Tables 3-2, 3-3, 3-4). Specific testosterone 6β-hydroxylase activity, which was not sexually differentiated in winter flounder microsomes (Table 3-3), was not correlated with any of the other monooxygenase activities examined. Turnover values between APDM, E_2 2-hydroxylase, and testosterone 6β-hydroxylase activities, which were elevated in mature female winter flounder, were positively correlated with each other. Only APDM and E_2 2-hydroxylase activities were positively correlated with each other, whether expressed as specific activites or turnover values. This consistent correlation between the two activities was also demonstrated in scup microsomes. As in scup, this could mean that the enzyme activities were catalyzed by co-regulated isozymes or by the same isozyme.

Discussion

Cytochromes P-450, b_5, and Reductase

As indicated in table 3-1, the specific content of cytochromes P-450 and b_5 and specific NADPH-cytochrome c reductase activity were in general lower in microsomes from female fish than from males. The specific content of cytochrome P-450 was also reported to be decreased in gonadally mature female brook trout and rainbow trout (Stegeman and Chevion, 1980), killifish (Koivusaari et al, 1981), and vendace (Lindstrom-Seppa, 1985). Hansson and Gustafsson (1981b) suggested that the high rates at which vitellogenic proteins are synthesized interfered with P-450 synthesis and monooxygenase activity in general in female fish. There may also be a contribution by vitellogenin toward microsomal proteins in mature females. Vitellogenin is synthesized in the liver and rapidly secreted into the bloodstream (Ho, 1987), but there are conflicting reports of its presence in livers from vitellogenic rainbow trout (Idler and Campbell, 1980; van Bohemen et al, 1981, 1982). If present in the hepatic microsomes, vitellogenin or its precursors could "dilute" levels of cytochromes P-450, b_5, and catalytic activities relative to total microsomal protein. An anti-vitellogenin immunoreactive protein of approximately 200 kD in hepatic microsomes from female killifish indicated the presence of vitellogenin. It is unknown, however, if vitellogenin is

present at levels that could significantly decrease monooxygenase activities per unit microsomal protein in pre-spawning female fish.

EROD and P-450E

The sex differences in EROD activity relative to microsomal protein in June scup and winter flounder reflect patterns of sex differences in aryl hydrocarbon hydroxylation reported in other teleosts. In gonadally mature cunner, rainbow trout, killifish, and vendace, AHH activity relative to protein was greater in males than in females, and these sex differences were not apparent in fish collected in the post-spawning period (Walton et al, 1978; Koivusaari et al, 1981; McKee et al, 1983; Lindstrom-Seppa, 1985). Gonadally immature scup did not display sex differences in EROD activity, although relatively slight sex difference was noted in immature winter flounder.

Microsomal specific EROD activity in mature scup collected in May was not sexually differentiated, and was low relative to levels in scup collected in June and at other times. The specific contents of microsomal P-450E were markedly lower in the May scup than in the scup collected in June, consistent with the difference in the specific EROD activity in these two samples. The scup collected in May had migrated within the past two months from their winter location in offshore waters (Morse, 1978). If the winter habitat had fewer inducing agents than the coastal waters of Cape Cod, EROD activities could have decreased to lower levels than are present in scup from in-shore waters. Alternatively, the low EROD activity and lack of sex difference may reflect a decrease in activity in both males and females immediately prior to spawning, such as reported for AHH in the vendace (Lindstrom-Seppa, 1985).

The specific content of P-450E and specific EROD activity demonstrate parallel sex differences in both winter flounder and June scup (Tables 3-2 and 3-5). Activities and isozymes normalized to spectral P-450 were also compared for sex differences. In immature winter flounder the levels of EROD and P-450E relative to spectral P-450 were not sexually differentiated. However, in mature winter flounder both the levels of P-450E and EROD activity relative to spectral P-450 were greater in males than in females. These differences suggested that upon maturation the levels of P-450E relative to total P-450 were suppressed in females, subsequently

reducing the microsomal turnover of 7-ethoxyresorufin. There was no sex difference in P-450E in May scup or June scup, although there was a trend toward higher levels of P-450E in the male than female June scup. This trend is reminiscent of the significant sex difference in the EROD turnover value in the June scup. It appears that the extent of sex differences in EROD activity was greater in winter flounder than in scup; the reason for this is unclear.

Previous papers have discussed the relationship between scup P-450E and the rat P-450c. Both isozymes are major aryl hydrocarbon hydroxylases, are strongly inducible by the MC-type inducers, and are immunochemically cross-reactive, although with apparently limited sequence homology (Klotz et al, 1986). They are suggested to have diverged from a common ancestral gene. Unlike teleost P-450E, the specific contents of both P-450c and the minor AHH catalyst, rat P-450d, were reported to be low and not sexually differentiated in untreated animals (Thomas et al, 1981; Waxman et al, 1985; Cresteil et al, 1986). Slightly higher (less than 2-fold) levels of AHH activity have been reported in male rats than in females (Warren and Bellward, 1978; Cresteil et al, 1986), but appear likely to have resulted from male-specific isozymes capable of AHH activity (Guengerich et al, 1982; Ryan et al 1984). It is apparent that P-450E is sexually differentiated in gonadally mature winter flounder and scup, imparting different levels of EROD activity in males and females. AHH activity was induced but not sexually differentiated in rats treated with a range of MC doses (Warren and Bellward, 1978). Therefore, gender does not appear to regulate P-450c in the rat, but has a marked effect on the levels of its teleost homolog. This represents a substantial difference between the regulation of two homologous isozymes.

Possible P-450A Activities: Testosterone 6β-Hydroxylation

Reconstituted scup P-450A catalyzed both testosterone 6β-hydroxylation and E_2 2-hydroxylation (Klotz et al, 1986), but in scup used in the present study only the latter activity was positively correlated with P-450A content. The turnover value for reconstituted testosterone 6β-hydroxylation was 0.8 nmol/min/nmol, greater than or equal to microsomal activities, suggesting that this isozyme was a major testosterone 6β-hydroxylase (Klotz et al, 1986). However, as P-450A content was not correlated with testosterone 6β-hydroxylation,

it might not contribute significantly to microsomal activity in scup. In rats, both reconstituted P-450c and RLM3 are steroid 6β-hydroxylases, yet neither contributes significantly toward activity in microsomes from BNF-treated rats or in microsomes expressing high levels of RLM3, respectively (Waxman, 1988). Possibly, this same phenomenon is being observed with P-450A in scup.

In winter flounder microsomes there was a positive correlation between P-450A content and testosterone 6β-hydroxylation, both per nmol P-450 and per mg protein. This indicates close co-variation between the P-450A homolog and the testosterone 6β-hydroxylase. Based on reconstitution of testosterone 6β-hydroxylase activity by scup P-450A (Klotz et al, 1986), the winter flounder P-450A homolog could be the microsomal 6β-hydroxylase, and sex differences in turnover values would be due to sexually differentiated levels of the P-450A homolog per nmol P-450 (Table 3-5). The P-450A homolog could be a significant microsomal 6β-hydroxylase in winter flounder, although its contribution toward microsomal 6β-hydroxylation in scup is unclear.

Possible P-450A Activities: E_2 2-Hydroxylation

Relative to microsomal activity, the turnover value for E_2 2-hydroxylation by reconstituted scup P-450A was low, 0.32 nmol/min/nmol (Klotz et al, 1986). However, E_2 2-hydroxylase activity by P-450A was not assayed until the isozyme had been in storage at -80°C for nearly two years. Because the P-450A sample had been in storage for so long, this value was thought to represent low levels of activity by partially denatured isozyme. The correlation analyses support P-450A contributing toward microsomal E_2 2-hydroxylation.

P-450A levels were also positively correlated with turnover values for microsomal APDM and E_2 2-hydroxylase activities, but the homolog and the activities were not correlated per mg protein. Furthermore, neither activity was correlated with specific testosterone 6β-hydroxylase activity, although they were correlated consistently with each other. This would suggest that in the winter flounder, APDM and E_2 2-hydroxylase activities were conducted by co-varying enzymes or by the same enzyme, and that this enzyme is not the equal to the testosterone 6β-hydroxylase. Therefore, in winter flounder, the APDM/E_2 2-hydroxylase catalyst(s) is unlikely to be a

P-450A homolog.

Because the relationship of fish isozymes with mammalian isozymes is of interest, scup P-450A has been compared with steroid metabolizing isozymes from rats. Previously, P-450A was shown to be immunochemically unrelated to rat cytochromes P-450c and P-450d, two MC-inducible proteins with testosterone 6β-hydroxylation activity (Wood et al, 1983; Klotz et al, 1986). Testosterone 6β-hydroxylation is also catalyzed by rat RLM3 (Cheng and Schenkman, 1983). RLM3 is a male-specific isozyme which, like P-450A, possessed a blue-shifted CO-ligated difference spectrum and low ECOD and AHH activities. However, unlike RLM3, catalytic activity by P-450A was stimulated by cytochrome b_5. In addition, RLM3, but not P-450A, was a testosterone 15α-hydroxylase. Anti-P-450A did not recognize RLM3 nor any other proteins in rat microsomes (J. Schenkman, pers. comm.), indicating that if they are functionally related, RLM3 and P-450A are structurally dissimilar.

The majority of microsomal 6β-hydroxylase activity has been ascribed to a PCN- or PB-inducible isozyme identified as PCN-E, or PB-2a (Waxman et al, 1985). Like P-450A and RLM3, reconstituted PCN-E catalyzed low rates of ECOD and AHH activities (Guengerich et al, 1982). PCN-E is also an E_2 2-hydroxylase, as indicated by antibody studies, although reconstitution of the activity is poor (Dannan et al, 1986b). PCN-E and P-450A have approximately the same molecular weights,and PCN-E has a slightly higher CO-ligated absorbance maximum (449 vs. 447.5 nm) (Guengerich et al, 1982). To date, PCN-E has not been tested for recognition by anti-P-450A.

There is sex-specific regulation of both the rat and scup isozymes. The specific contents of RLM3 and PCN-E were greater in microsomes from adult male rats than from adult females. RLM3 levels, which are also strain dependent, are low in immature rats and increase in males at puberty while remaining low in females (Bandiera et al, 1986; McClellan-Green et al, 1987). PCN-E levels are maintained in the adult male rat through neonatal imprinting by androgens, and can be sustained even in gonadectomized adults without further testosterone input. In normal females, PCN-E levels decrease at puberty (Waxman et al, 1985; Dannan et al, 1986a). The patterns of sexual differentiation of P-450A and the winter flounder homolog were

unlike the regulatory patterns of rat RLM3 and PCN-E. Unlike the rat isozymes, the specific content of P-450A was not sexually differentiated in microsomes from mature scup and winter flounder. However, the P-450A homolog levels were greater relative to total spectral P-450 in female winter flounder than in males. Compared to rat RLM3 and PCN-E, then, P-450A appears to be a functionally similar protein with a distinctly different pattern of sexual regulation.

APDM and E_2 2-Hydroxylation

The rate of aminopyrine N-demethylation is approximately 3.5 times greater in microsomes from untreated male rats than from females, and is inducible in both sexes by PB (Kato, 1974; Kato and Onada, 1980). APDM is efficiently metabolized by male specific P-450 RLM5 and by PB-inducible P-450 isozymes (Guengerich et al, 1982; Schenkman et al, 1987). APDM activity in teleost microsomes was not induced by MC-type inducers, nor inhibited by anti-P-450E (Binder et al, 1985, Goddard et al, 1987, Kloepper-Sams et al, 1987). Therefore, this activity represents monooxygenation catalyzed by teleost isozymes other than P-450E, but possibly related to PB-inducible or male-specific rat isozymes.

Like APDM, E_2 2-hydroxylase activity in rats was greater in microsomes from untreated male than from females (Jellinck and Lucieer, 1965). Antibody studies indicated that this sex difference in rats is due to E_2 2-hydroxylation being catalyzed by two male specific isozymes, RLM5 and PCN-E (Dannan et al, 1986b). As such, this activity is inducible in female rats by both testosterone and PCN (Namkung et al, 1985; Dannan et al, 1986b). In teleost microsomes E_2 2-hydroxylation was not inhibited be anti-P-450E (Snowberger and Stegeman, 1987), precluding catalysis by a counterpart to rat P-450c. Possibly, E_2 2-hydroxylation in teleosts represents catalysis by teleost isozymes related to male specific, and/or PCN-inducible forms.

The good correlation between APDM activity and E_2 2-hydroxylation in scup and January winter flounder indicated that metabolism could be catalyzed by the same enzyme or by enzymes present at similar levels. The sex differences in the turnover values for both APDM and E_2 2-hydroxylation in winter flounder also suggested that the level or the activity of the responsible catalyst(s) in this species was sexually regulated. In these respects, the speculative

APDM/E_2 catalyst in winter flounder resembles male-specific rat RLM5. RLM5 efficiently catalyzed both APDM and E_2 2-hydroxylation, with little or no testosterone 6β-hydroxylase activity (Ryan et al, 1984; Morgan et al, 1985b; Schenkman et al, 1987). RLM5 is also sexually differentiated; high levels of the isozyme in adult male rats are imprinted by neonatal testosterone and maintained by circulating testosterone in adulthood. However, RLM5 is characterized primarily by testosterone 2α- and 16α-hydroxylase activity. A testosterone metabolite of winter flounder microsomes did co-migrate with 2α-OH-testosterone, but upon elution and rechromatography in another solvent system this metabolite did not migrate with the standard. A product that co-migrated with 16α-OH-testosterone was present only in minor quantities. Therefore, two prominent characteristics of RLM5 were not apparent in winter flounder microsomes.

Rat PCN-E has been discussed already as a possible homolog of scup P-450A based on its testosterone 6β-hydroxylase activity. In addition, PCN-E is an E_2 2-hydroxylase and AP demethylase (Guengerich et al, 1982; Dannan et al, 1986b). If a PCN-E homolog is present in teleost microsomes, it could contribute toward APDM, E_2 2-hydroxylase, and testosterone 6β-hydroxylase activities. However, based on correlation analyses, a teleost homolog to PCN-E is unlikely to be the primary catalyst of all three activities within a species.

Recently, P-$450_{C-M/F}$, an isozyme unrelated to RLM5 and PCN-E, was purified from untreated males and female rats (Sugita et al, 1988). P-$450_{C-M/F}$ did not metabolize testosterone efficiently, but catalyzed E_2 2-hydroxylation and both EM and benzphetamine demethylation. More information will be needed to determine if P-$450_{C-M/F}$ resembles the catalyst(s) conducting APDM and E_2 2-hydroxylase activities in winter flounder microsomes.

Conclusions

At least four monooxygenases were sexually differentiated in microsomes from gonadally mature scup and winter flounder. None of the sex differences were apparent in gonadally immature animals, indicating that this phenomenon is associated exclusively with biochemical changes occurring at spawning. Catalytic activities relative to microsomal protein were often decreased in females, suspected to be due partially to proteins such as vitellogenin in the

microsomes. Sex differences relative to cytochrome P-450 were also observed, especially in winter flounder, and appeared to reflect modulation of specific isozymes within the P-450 population. Suppressed EROD activity in both female winter flounder and scup apparently resulted from reduced levels of P-450E or its homolog. The turnover values for APDM, testosterone 6β-hydroxylation and E_2 2-hydroxylation were, if sexually differentiated, greater in females than in males. Elevated testosterone 6β-hydroxylase activity in females is proposed to result from increased levels of the P-450A homolog, which shows physical, catalytic, and regulatory similarities with rat RLM3, but does not cross-react immunochemically. It is proposed that APDM and E_2 2-hydroxylase activities are catalyzed by other isozymes that were sexually regulated in winter flounder. The catalytic properties of the winter flounder APDM and/or E_2 2-hydroxylase catalyst(s) resemble those of rat isozymes RLM5, PCN-E, and recently purified $P\text{-}450_{C\text{-}M/F}$; further analysis is necessary to determine if there are homologous proteins.

In rats, both exogenous and endogenous factors are already known to regulate cytochrome P-450 isozyme (see Introduction). Rat P-450c and teleost P-450E are both inducible by MC-type compounds, but it appears from this work than in teleosts P-450E and P-450E-metabolized activities are also regulated by sex. Other isozymes could be sexually regulated as well, as evidenced by sex differences in activities not catalyzed by P-450E. The development in gonadally mature female fish of sex differences in P-450 isozymes and monooxygenase activities indicated possible regulation by female specific factors, including the sex steroid hormone estradiol. This possibility was investigated in winter flounder, and is reported in the next chapter.

CHAPTER 4: EFFECTS OF E_2 TREATMENT ON MONOOXYGENASE ACTIVITIES IN WINTER FLOUNDER

Introduction

Previous studies had indicated that the steroid hormone E_2 was a possible effector of sex differences in hepatic monooxygenases in freshwater trout (Hansson, 1982; Stegeman et al, 1982). As shown in Chapter 3, winter flounder microsomal monooxygenases exhibited strong sex differences that were most pronounced in the period preceding spawning. To determine if E_2 was also regulatory in marine species, the effect of the hormone on monooxygenation was tested in immature winter flounder. The availability of winter flounder, extensive background information on other effects of E_2 treatment, and the strong sex differences in monooxygenases in adults made them favorable candidates for study.

E_2 treatment of winter flounder was undertaken in May, approximately three months after the peak spawning period. Normally, by May winter flounder have already vacated coastal waters for deeper, cooler off-shore areas, so fish were collected in early April and held for more than a month until treatment. The dosage regime was based on the reported success of other E_2-treatment regimes at inducing vitellogenesis in winter flounder and at altering cytochrome P-450 in trout (Campbell and Idler, 1976; Stegeman et al, 1982). A range of E_2 dosages was tested in winter flounder. As will be shown, even the lowest doses effected changes in monooxygenase activities, suggesting that the winter flounder monooxygenase system is quite sensitive to E_2.

Microsomes from the experimental winter flounder were subjected to numerous assays. For each parameter, the magnitude of the difference between control and E_2-treated fish was compared to that between mature male and female winter flounder to determine if E_2 treatment might be reproducing a sex difference. In some cases sex differences were not reproduced, or were only partially expressed. This suggested that factors other than or in addition to E_2 regulate monooxygenases in mature females, and is considered in the Discussion.

Materials and Methods

Chemicals

Chemicals were obtained as in Chapter 3.

Animals

Winter flounder were collected by otter trawl from Cleveland

Ledge Channel in Buzzards Bay on April 1, 1986. Dissection of some fish at the time of collection showed that the majority were gonadally regressed. Gonads were small compared to those in spawning winter flounder, and only a few contained eggs or milt. Live fish were transferred to the Shore Laboratory at WHOI and maintained in an aquarium under the natural light cycle with ambient (12-15°C) flowing seawater and clean sediment for at least one month. They were fed chopped squid ad libitum 2-3 times per week.

Experimental Treatment

In early May 1986, four of the held winter flounder were sacrificed and examined to confirm that hepatic monooxygenase activities were not sexually differentiated. Twenty remaining winter flounder (300-600 g body weight) were then transferred into two water tables with clean sediment (2.4 m x 0.7 m x 13 cm; 60 gal). The water tables were within the Shore Laboratory and received water from the same source as the aquarium from which the winter flounder were tranferred. Each water table was partitioned with a screen into two halves such that there were four separated groups of fish. Winter flounder of mixed sex were used as it is difficult to distinguish sex in regressed fish by external appearances. Seawater flowed into the tank of control fish, then passed sequentially into tanks I, II, and III, which contained fish that would receive successively higher doses of E_2. Fish were acclimated for 5 days before beginning the experiment.

E_2 (Sigma) was dissolved in dimethylsulfoxide (DMSO) to 2, 20, or 100 mg/ml. On days 0 and 3, winter flounder were injected i.p. with 0.5 ml/kg of the appropriate solution to receive 1, 10, or 50 mg/kg E_2. Control fish were injected with 0.5 ml/kg DMSO. There did not appear to be significant leakage from injection sites, and the direction of water flow minimized chances that E_2 from a highly dosed fish would enter a tank of fish receiving lower doses. There were no mortalities during the treatment period. The winter flounder were sacrificed on day 7.

Analysis of E_2-Treated Winter Flounder

Blood was collected from each individual immediately prior to sacrifice, and was later centrifuged for 20 minutes at 1500 x g. The plasma supernate was stored at -80°C until analysis. Plasma from these fish and from mature winter flounder previously collected in

November 1983 was analyzed for E_2 and testosterone by radioimmunoassay at the laboratory of Dr. P. Thomas, U. of Texas at Port Aransas, by the method described in Stegeman et al (1982).

Gonads and livers from the experimental fish were weighed at the time of sacrifice. Hepatic microsomes were prepared and assayed for cytochrome P-450 and monooxygenase activities by methods described in chapter 3.

Statistics

Results of analysis are expressed as means ± standard deviations. Means were analyzed by ANOVA, and if statistical differences were found, the means were analyzed by Dunnett's test (Zar, 1974) to determine where the differences lay. Significance of correlation coefficients was determined by Student's t-test.

Results

Untreated, Regressed Winter Flounder

As noted in the Methods section, examination of several winter flounder at the time of collection in April showed that almost all were gonadally regressed. Literature reports on sex differences in fish and archived data from this laboratory indicated that by May the monooxygenase activities in gonadally regressed female winter flounder should have returned to the levels of immature fish. To confirm this, four of the animals reserved for experimentation were sacrificed and examined. Data from these winter flounder killed in early May were compared to archived data on winter flounder examined in early June of 1985. It was readily apparent that monooxygenase activities were unlikely to be sexually differentiated in winter flounder in May.

Altogether, three female and one male winter flounder were examined. The mean HSI and GSI of the regressed female winter flounder were 3- and 9-times lower, respectively, than in the mature January females (Table 3-1; Table 4-1). The mean HSI and GSI of females collected in May were also as low as those in June winter flounder. This would indicate that the females were not engaged in vitellogenesis or spawning, as would be expected from the season (Bigelow and Schroeder, 1953).

The specific content of microsomal cytochrome P-450 or cytochrome b_5 in the regressed females collected in May was 5-6 times greater than in the mature females, but also somewhat greater than in the fish

TABLE 4-1: MICROSOMAL CHARACTERISTICS OF UNTREATED REGRESSED WINTER FLOUNDER

	May, 1986		June, 1985	
	Male N = 1	Female N = 3	Male N = 4	Female N = 4
HSI[a]	0.94	0.73 ± 0.14	0.93 ± 0.27	1.72 ± 1.25
GSI[b]	0.61	1.95 ± 0.54	0.33 ± 0.22	2.04 ± 0.61
Cytochrome P-450				
nmol/mg	1.83	1.63 ± 0.54	0.55 ± 0.30	0.36 ± 0.18
Cytochrome b_5				
nmol/mg	0.16	0.10 ± 0.00	0.04 ± 0.02	0.05 ± 0.01
EROD				
nmol/min/mg	6.52	4.47 ± 0.74	0.97 ± 0.52	0.68 ± 0.55
nmol/min/nmol	3.56	3.00 ± 1.25	1.79 ± 0.48	1.40 ± 0.68

[a]HSI = hepatosomatic index (liver weight/body weight) x 100
[b]GSI = gonadosomatic index (gonad weight/body weight) x 100

examined in June. Neither component was sexually differentiated in the June winter flounder. EROD activities per mg microsomal protein and per nmol cytochrome P-450 were also greater in the females collected in May than in the mature January female flounder and the regressed females sampled in June. Contaminant inducing agents at the sampling site, Buzzards Bay, could have been responsible for the relatively high levels of EROD activity in the May fish (Stegeman et al, 1987). However, the site alone would not be expected to inhibit sex differences as EROD activities in winter flounder collected in November from the same site were lower in adult females than in adult males (Table 3-2). In the winter flounder examined in June there was no indication of the greater than 30-fold sex difference in EROD activity observed in gonadally mature fish. It therefore seemed unlikely that EROD activities would be sexually differentiated in the other as yet unsacrificed May winter flounder. Based on literature reports and these results, the remaining gonadally regressed winter flounder were deemed acceptable to study the effects of E_2 treatment on hepatic monooxygenase activities.

E_2 Treated Winter Flounder

Plasma steroids: Mean plasma levels of E_2 were less than 1 ng/ml in control winter flounder, and were elevated in the E_2-treated fish (Table 4-2). In group I, receiving 1 mg/kg of E_2, plasma E_2 levels ranged from 35 to 156 ng/ml. As will be discussed, this is within the range of physiological E_2 levels reported for plaice (Wingfield and Grimm, 1977), and slightly greater than in winter flounder collected in November (16-42 ng/ml). In groups II and III, dosed with 10 and 50 mg/kg E_2 respectively, average plasma E_2 levels exceeded 250 ng/ml. In one individual from group III, however, the plasma E_2 level was only 13.7 ng/ml. Other parameters measured for this individual were not notably different from othermembers of group III, and this individual was included in characterizing this group. The plasma sample was analyzed twice, yielding similar results, and it seemed unlikely that the fish had excreted the large dose of E_2 within 3 days of the last injection. However, I do not know why plasma E_2 was so low in this individual.

Plasma testosterone levels were low in the control groups and in all of the treatment groups (Table 4-2). The entire range of

TABLE 4-2: PLASMA STEROIDS AND HSI OF CONTROL AND E_2-TREATED WINTER FLOUNDER

Group	E_2 Dose mg/kg	N	Estradiol ng/ml	Testosterone ng/ml	HSI
Control	--	3[c]	0.30 ± 0.20	0.35 ± 0.15	0.74 ± 0.09[d]
I	1	4	88 ± 53[a]	0.73 ± 0.66	1.13 ± 0.28[a]
II	10	6	258 ± 61[a]	0.36 ± 0.26	1.17 ± 0.32[a]
III	50	5	405 ± 238[b]	0.19 ± 0.02	1.26 ± 0.24[b]

[a,b]Significantly different from control group, [a] $P<0.05$; [b] $P<0.01$
[c]Plasma samples from 3 control fish were analyzed for steroid content. There were 5 animals in the control group.
[d]N = 5

testosterone levels (from 0.18 to 1.72 ng/ml) corresponded with the lowest levels of testosterone in plasma of post-spawning male and female winter flounder (less than 2 ng/ml; Campbell et al, 1976).

HSI and GSI: All E_2 dosages were associated with slight but significantly increased mean hepatosomatic indices in the treated winter flounder (Table 4-2). In some of the E_2-dosed winter flounder, the livers had an orange tint. In gonadally mature female winter flounder, the livers are often tinted orange, and in general are more friable than those of the males. The texture and firmness of the livers from groups I-III were not notably different from those of the control group.

The gonadosomatic indices of male winter flounder were not affected by E_2 treatment. There were only two female winter flounder within the control group, too few for statistical comparisons with the GSI of the female E_2-treated fish. However, the GSI of the E_2-treated winter flounder were not different from those of the untreated, regressed females sacrificed only two weeks earlier (Table 4-1). Therefore, E_2 treatment did not appear to affect the GSI of either sex.

Microsomal cytochromes P-450 and b_5, and reductase: The specific content of microsomal cytochrome P-450 in winter flounder treated with E_2 was significantly lower than in the control group (Table 4-3). For these and other parameters, there was not a significant difference within groups between male and female experimental animals, so the sexes were pooled to yield the group mean. The response to E_2 was not dose-dependent, as the specific content of cytochrome P-450 was not differentiated between the experimental groups. Only one individual demonstrated cytochrome P-420, an indication of degraded cytochrome P-450, and it was present in relatively minor amounts. The mean CO-bound reduced absorbance maximum of the control group was 448.7 ± 0.2 nm, and was not significantly different from that of any of the E_2-treated groups.

The specific content of microsomal cytochrome b_5 was also significantly decreased in all of the E_2-treated groups (Table 4-3). As with cytochrome P-450, the decrease in cytochrome b_5 was not dose dependent. The mean specific content of cytochrome b_5 in each group of E_2-treated winter flounder was approximately 50%

TABLE 4-3: MICROSOMAL COMPONENTS OF CONTROL AND E_2-TREATED WINTER FLOUNDER

Group	N	Cytochrome P-450 nmol/mg	Cytochrome b_5 nmol/mg	NADPH-cyto. c Reductase nmol/min/mg
Control	5	1.39 ± 0.11	0.096 ± 0.018	38.4 ± 4.3
I	4	0.96 ± 0.42[b]	0.050 ± 0.014[b]	31.0 ± 6.4[a]
II	6	0.66 ± 0.16[b]	0.045 ± 0.012[b]	29.0 ± 2.8[b]
III	5	0.68 ± 0.25[b]	0.055 ± 0.018[b]	36.4 ± 6.4

Experimental groups are as described in Table 4-2.
[a,b]Significantly different from control group, [a] $P<0.05$; [b] $P<0.01$

of the mean of the control group.

NADPH-cytochrome c reductase activity was significantly decreased in groups I and II to approximately 75°/o of the control values (Table 4-3). This was less pronounced than the 50°/o decreases observed for microsomal cytochromes P-450 and b_5 in these groups. Mean reductase activity in group III was not different from that of the control group, although both cytochromes P-450 and b_5 were substantially decreased in this group.

Xenobiotic metabolism: Ethoxyresorufin O-deethylase specific activity was decreased in all of the experimental groups (Table 4-4). This decrease was not dose-dependent; mean EROD activity was decreased by approximately 60°/o in each of the E_2-treated groups. In addition, turnover values for EROD activity were decreased in each treatment group to 40°/o of the mean activity in the control group. Again, this decrease was not dose-dependent.

Aminopyrine N-demethylase activity per mg microsomal protein was significantly decreased in all experimental groups to 50-60°/o of control values (Table 4-4). Mean APDM activities were not significantly different between the E_2-treated groups. The turnover values for APDM were unchanged by E_2 treatment. The turnover values suggest that after week-long exposure to E_2 the efficiency at which the microsomal cytochrome P-450 population metabolized APDM was unaltered while the efficiency for EROD metabolism was decreased.

Steroid metabolism: Testosterone 6β-hydroxylation per mg microsomal protein was significantly decreased in groups II and III, which were treated with the highest doses of E_2 (Table 4-5). However, levels of activity relative to microsomal cytochrome P-450 were unaltered in any group by E_2 treatment. In contrast, turnover values for this activity were significantly greater in mature female winter flounder than in adult males. The calculated standard deviations were high, representing up to 100°/o of mean activity.

Levels of specific E_2 2-hydroxylase activity were decreased in microsomes from E_2-treated winter flounder, consistent with the theory that E_2 suppressed the same activity in the adult female. The turnover values for E_2 2-hydroxylation were also decreased in the fish treated with E_2. However, in mature winter flounder turnover values either were not sexually differentiated or were

TABLE 4-4: XENOBIOTIC METABOLISM IN CONTROL AND E_2-TREATED WINTER FLOUNDER

Group	N	Ethoxyresorufin O-deethylation		Aminopyrine N-demethylation	
		unit[c]/mg	unit/nmol P-450	unit/mg	unit/nmol P-450
Cont.	5	4.08 ± 0.57	2.95 ± 0.45	1.52 ± 0.49	1.09 ± 0.36
I	4	1.79 ± 1.12[b]	1.66 ± 0.80[a]	1.01 ± 0.38[a]	1.09 ± 0.17
II	6	1.19 ± 0.60[b]	1.73 ± 0.71[b]	0.59 ± 0.25[b]	0.92 ± 0.36
III	5	1.39 ± 0.71[b]	2.02 ± 0.79[a]	0.84 ± 0.46[a]	1.17 ± 0.28

Experimental groups are as described in Table 4-2

[a,b]Significantly different from control group, [a] $P<0.05$; [b] $P<0.01$

[c]unit = nmol EROD product (resorufin) or APDM product (HCHO) produced per minute

TABLE 4-5: MICROSOMAL STEROID METABOLISM IN CONTROL AND E_2-TREATED WINTER FLOUNDER

Group	N	Testosterone 6β-hydroxylation		Estradiol 2-hydroxylation	
		unit[c]/mg	unit/nmol P-450	unit/mg	unit/nmol P-450
Control	5	0.034 ± 0.006	0.025 ± 0.005	0.354 ± 0.038	0.257 ± 0.037
I	4	0.021 ± 0.005	0.028 ± 0.022	0.163 ± 0.061[b]	0.174 ± 0.017[a]
II	6	0.013 ± 0.012[b]	0.021 ± 0.021	0.122 ± 0.059[b]	0.176 ± 0.056[b]
III	5	0.021 ± 0.014[a]	0.037 ± 0.032	0.119 ± 0.029[b]	0.183 ± 0.045[b]

Experimental groups are as described in Table 4-2
[a,b]Significantly different from control group, [a] $P<0.05$; [b] $P<0.01$
[c]unit = nmol 6β-OH-testosterone or 2-OH-E_2 produced per minute

slightly greater in females than in males (Table 3-4). The observation that E_2 treatment decreased E_2 2-hydroxylation turnover values to approximately 70°/o of the control values suggested that the response of the immature winter flounder to E_2 was not entirely reproducing the response of the adult. This will be considered further in the Discussion.

Isozyme composition: Microsomal levels of P-450E and P-450A equivalents were quantitated by immunoblotting and densitometric scanning. In all groups of E_2-treated winter flounder, specific P-450E levels were significantly decreased to approximately 60°/o of control levels (Table 4-6). As in the analyses of microsomal monooxygenase activity, the response to E_2 was not dose-dependent. In contrast to the response of the P-450E homolog, the specific content of P-450A equivalents was unchanged by E_2 treatment. The levels of P-450E equivalents were not different, following normalization to spectral cytochrome P-450, between the control group and the E_2-treated groups. Statistically, the level of P-450A per pmol cytochrome P-450 was not differentiated by E_2 treatment, but there seemed to be a trend toward elevated homolog levels.

Discussion

Plasma Steroids, HSI and GSI

Plasma steroids: The rate of E_2 release from DMSO was unknown, but DMSO is known to enhance absorption across membranes. Presumably, in the winter flounder experiments E_2 was released rapidly from DMSO and absorbed across the intraperitoneal cavity. In goldfish, plasma E_2 concentrations peaked within one hour following a single intraperitoneal injection of E_2 in saline or peanut oil, then declined steadily for at least eight days (Pankhurst et al, 1986). It is presumed that at the time of sacrifice plasma E_2 concentrations in the experimental winter flounder were declining from an unknown peak value achieved immediately following injections.

Plasma levels of E_2 in the steroid treated winter flounder were, at the time of sacrifice, greater than or equal to reported physiological concentrations of E_2 in flatfish. Plasma E_2 levels in female winter flounder collected in November from Buzzards Bay ranged from 16 to 42 ng/ml. The peak spawning period for winter flounder in these waters is in February (Bigelow and Schroeder, 1953),

TABLE 4-6: MICROSOMAL P-450E AND P-450A CONTENT IN CONTROL AND E_2-TREATED WINTER FLOUNDER

Group	N	P-450E equivalents		P-450A equivalents	
		unit[c]/mg	unit/nmol	unit/mg	unit/nmol
Control	5	0.43 ± 0.08	0.31 ± 0.04	0.56 ± 0.09	0.41 ± 0.07
I	4	0.27 ± 0.12[a]	0.27 ± 0.04	0.55 ± 0.12	0.73 ± 0.51
II	6	0.19 ± 0.07[b]	0.29 ± 0.08	0.47 ± 0.11	0.74 ± 0.19
III	5	0.22 ± 0.12[b]	0.31 ± 0.11	0.49 ± 0.10	0.78 ± 0.24

Experimental groups are as described in Table 4-2

[a,b]Significantly different from control group, [a] $P<0.05$; [b] $P<0.01$

[c]unit = P-450E or P-450A equivalents, based on standards included on the same blot, per mg protein or per nmol P-450

and in general steroid hormone levels do not peak until immediately before spawning (Fostier et al, 1983). Therefore, plasma E_2 levels in female winter flounder collected in November were probably below peak physiological levels. There does not appear to be information on the cycle of plasma E_2 levels in winter flounder, but in plaice, a closely related species, E_2 concentrations peaked at 162 ± 10 ng/ml in pre-spawning females (Wingfield and Grimm, 1977). This maximum was close to the range of plasma E_2 in group I, the winter flounder dosed with only 1 mg/kg E_2 (35-156 ng/ml). In groups II and III, which were dosed with 10 and 50 mg/kg E_2, respectively, plasma E_2 levels were in excess of 250 ng/ml. This was of concern, both for being excessively elevated compared to known physiological levels of plasma E_2 and because high doses of E_2 were suggested to evoke hepatic necrosis in another teleost, the red grouper (Epinephelus akaara) (Ng et al, 1985). Pharmacological doses of E_2 were employed to increase the likelihood of observing E_2-mediated effects on hepatic monooxygenase activities. However, the affect of large doses of E_2 on hepatic tissues in winter flounder is not reported.

Plasma testosterone levels were low in both control and treatment groups. In mature female teleosts, including flounder, testosterone levels are elevated as this hormone is the precursor for both E_2 and the major androgen 11-ketotestosterone (Campbell and Idler, 1976; Wingfield and Grimm, 1977). In studies of brook trout and killifish, E_2 injections were associated with elevated plasma testosterone (Stegeman et al, 1982; Stegeman, Singh et al, unpublished). This phenomenon was suggested to represent positive feedback in response to low levels of steroid hormones (Stegeman et al, 1982). In adult fish, steroid hormone regulation by positive feedback appears restricted to the beginning of the plasma steroid surge during the prespawning phase of the reporductive cycle (Peter and Crim, 1979). That testosterone levels were unchanged in E_2-treated winter flounder indicates that at the time of the experiment, there was no evidence of positive feedback stimulation of steroid secretion.

HSI and GSI: The increased hepatosomatic indices of E_2-treated winter flounder were in agreement with literature reports of E_2 elevating the HSI of other teleost species including flounder (Emmersen et al, 1979), brook trout (Stegeman et al, 1982), and

rainbow trout (Haux and Norberg, 1985). Exogenous E_2 is well known to stimulate vitellogenesis, or yolk protein synthesis, in teleost livers. In two studies, hepatic lipid and protein content were increased and glycogen stores were depleted following E_2 treatment of both male and female fish, indicating mobilization of resources for lipophophoprotein synthesis (de Vlaming et al, 1977; Emmersen et al, 1979). In other studies, vitellogenin was detected in the plasma but not the liver of E_2-treated fish, suggesting that yolk proteins were rapidly secreted into the bloodstream after synthesis (Yaron et al, 1980; van Bohemen et al, 1982; Ng et al, 1985). Positive identification of the liver as the source of vitellogenin was achieved with tracer studies using radiolabeled amino acids in cod (Plack et al, 1971) and $^{33}PO_4$ in winter flounder (Campbell and Idler, 1976). The plasma of the E_2-treated winter flounder used here was not analyzed for vitellogenin, but it is likely that the HSI were elevated because the livers were synthesizing or preparing to synthesize this protein in a classic response to E_2 treatment.

The gonadosomatic indices of the E_2-treated winter flounder were not different from the control group. Previously, E_2 treatment did not affect the GSI in goldfish (de Vlaming et al, 1977), stinging catfish Heteropneustes fossilis (Sundararaj and Nath, 1981), brook trout (Stegeman et al, 1982), or the cyprinid fish Mirogrex terrae-sanctae (Yaron et al, 1980). E_2 treatment elevated plasma vitellogenin in hypophysectomized winter flounder, but pituitary extracts were required to elevate the GSI and stimulate incorporation of yolk proteins into the ovaries (Campbell and Idler, 1976). The pituitary extracts contained gonadotropic hormone (GtH), which stimulates gonadal secretion of sex steroids and the development of gametes. The regulation of GtH is not fully understood, but sex steroids can influence GtH secretion through both positive and negative feedback at the pituitary and the hypothalamus (review: Goos, 1987). In the E_2-treated winter flounder, both the lack of gonadal growth and the unchanged testosterone levels indicated that GtH secretion was not stimulated.

Microsomes

Cytochromes P-450, b_5, and reductase: The decreased specific content of microsomal cytochromes P-450 and b_5 in E_2-treated

winter flounder was in agreement with observations made in similar studies of E_2 in rainbow trout and brook trout (Hansson and Gustafsson, 1981b; Stegeman et al 1982). That the decreases were not dose-dependent may indicate that the lowest dosage of E_2 injected (1 mg/kg) was sufficient to achieve the maximal extent to which cytochrome P-450 levels would decrease. Cytochrome P-420 was absent, even from groups dosed with the highest amount of E_2, suggesting that experimental treatment did not promote cytochrome P-450 denaturation to this form; heme loss, however, would not have been detected. Therefore, high E_2 doses did not appear to have adverse effects on the hepatic functions studied here.

Qualitatively, E_2-induced decreases in microsomal cytochrome P-450 and cytochrome b_5 resembled suppression of these microsomal components in mature female winter flounder. This supports the hypothesis that in female winter flounder E_2 is in some manner down-regulating cytochromes P-450 and b_5. Quantitatively, the differences between the levels of these microsomal components in the experimental fish and the control fish were similar to the sex differences between microsomal components in winter flounder collected in November (Table 3-1). The extent of the decreases of cytochromes P-450 and b_5 in the experimental fish were less than the sex differences observed between mature fish collected in January, due perhaps to the longer duration of elevated plasma E_2 in adult females. Longer exposure to E_2 might have accentuated suppression of these microsomal components in the experimental winter flounder.

Microsomal NADPH-cytochrome c reductase activity was decreased in two of the three treatment groups. Stegeman et al (1982) did not observe a change in this activity in microsomes from steroid-treated brook trout. Possibly, reductase activity in winter flounder was more sensitive to E_2 than in brook trout. Statistically, reductase activity was not sexually differentiated in microsomes from mature winter flounder (Table 3-1). E_2 treatment may have decreased reductase activity in immature flounder via channels normally unavailable in mature fish, due perhaps to the hormonal mileau of adult females.

Xenobiotic metabolism: EROD activity per mg microsomal protein was suppressed by E_2 treatment to approximately 35^{o}/o of levels of

activity in control fish. This decrease in EROD activity following E_2 treatment supported the hypothesis that this hormone down-regulates EROD activity in mature female winter flounder. However, in microsomes from mature female winter flounder collected in November and January, EROD activity represented a much lower percentage, only 15°/o and 3°/o, respectively, of activity in microsomes from males. EROD turnover values in E_2-treated fish were decreased to 60°/o of control values. Again, the extent to which the turnover values for EROD activity were decreased in E_2-treated winter flounder was less than the extent of suppression in sexually mature female winter flounder (40°/o and 11°/o of activity in males collected in November and January, respectively). This would suggest that E_2 treatment alone may be insufficient to fully account for sex differences in EROD activity in mature winter flounder.

APDM activity per mg microsomal protein was decreased by E_2 treatment to approximately 55°/o of control levels. In microsomes from mature female winter flounder, specific APDM activity represented 57°/o of activity in mature males. This suggests that E_2 could be responsible, in part, for the decreased specific APDM activity in gonadally mature female winter flounder. Turnover values for APDM were unchanged in immature fish by E_2 treatment, although turnover values were greater in gonadally mature female winter flounder than in males. Therefore, E_2 treatment suppressed APDM specific activity, but the efficiency of the P-450 pool for metabolizing aminopyrine was unaltered. This contrasts with the decreased EROD turnover values following E_2 treatment, which was qualitatively similar to sex differences in EROD activity. Possibly, factors other than or in addition to week-long exposure to E_2 would be required to elevate turnover values for APDM activity in winter flounder.

Steroid metabolism: Testosterone 6β-hydroxylase activity per mg protein was decreased in microsomes from E_2-treated winter flounder. This activity was also decreased in juvenile brook trout and juvenile rainbow trout following E_2 treatment (Stegeman et al, 1982; Hansson, 1982). Hansson and Gustafsson (1981b) reported lower rates of specific 6β-hydroxylation in mature female rainbow trout than in males, and results of their E_2 experiment suggested that E_2 suppressed activity in mature female rainbow trout. In winter

flounder, though, E_2 suppressed specific testosterone 6β-hydroxylation, although this activity was not sexually differentiated in mature fish (cf Table 3-3). Microsomal turnover values for testosterone 6β-hydroxylation were not differentiated by E_2 treatment, yet in mature winter flounder turnover values were 5 times greater in females than in males. The high turnover value in mature females may have compensated for general decreases in monooxygenase activities expressed per mg protein such that specific testosterone 6β-hydroxylation was not sexually differentiated. This is suggested by observing that in gonadally mature female flounder, all of the monooxygenase activities assayed were decreased per mg protein save for testosterone 6β-hydroxylation. Possibly, decreased specific testosterone 6β-hydroxylase activity per mg protein may be apparent in E_2-treated winter flounder because turnover values for this activity were not increased as they were in gonadally mature females.

E_2 2-hydroxylase activity per mg microsomal protein was decreased in E_2-treated winter flounder, resembling the sexually differentiated activity in mature winter flounder. However, E_2 2-hydroxylase activity per nmol P-450 was also significantly decreased by E_2 treatment, although turnover values for E_2 2-hydroxylation by mature female winter flounder were slightly greater than or equal to males. It is a concern that E_2 injected into the experimental fish might have been present in the microsomes, possibly decreasing measurements of E_2 2-hydroxylation by substrate inhibition or by diluting the radiolabeled substrate [2-^{3}H]E_2. However, the decreases in E_2 2-hydroxylase activity were not dose-dependent although E_2 dosages ranged from 1 to 50 mg/kg and plasma E_2 levels ranged from 35-660 ng/ml. Dilution of the radiolabled substrate, then, appears unlikely. Inhibition of E_2 2-hydroxylation in microsomes from untreated winter flounder was observed when substrate concentration exceeded exceeded 50μM E_2 (Figure 4-1). It was calculated that 4 times the amount of injected E_2 would have to have been present in hepatic microsomes from group I to contribute an inhibitory quantity of exogenous E_2. It should be noted that although cytosolic E_2 binding sites were identified in the winter flounder liver (Sloop et al, 1984), binding by the microsomal fraction was not reported. It seems unlikely, then, that E_2 injected into

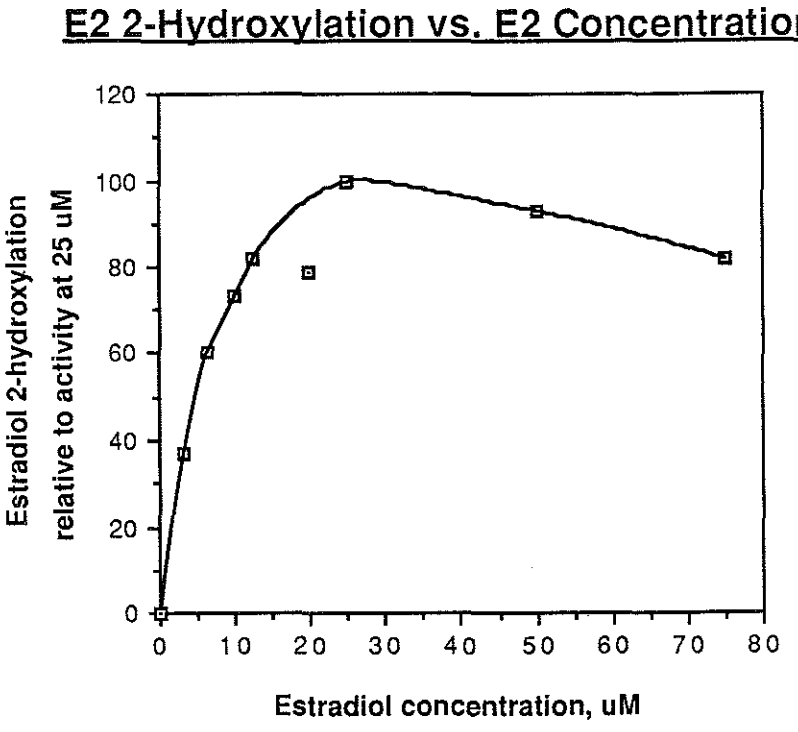


Figure 4-1. Microsomal E_2 2-hydroxylase activity as a function of substrate concentration. Activity in winter flounder microsomes was assayed as in Snowberger and Stegeman, 1987, except that [2-^{3}H]E_2 concentra- tions ranged from 3 to 75 uM. Mean activities of duplicate assays are presented relative to activity at 25 uM [2-^{3}H]E_2.

the winter flounder was present in microsomes and affecting E_2 2-hydroxylase activity.

Therefore, decreased turnover values for microsomal E_2 2-hydroxylation in E_2-treated winter flounder appear to indicate decreased efficiency of the cytochrome P-450 population for this activity. Yet, turnover values for E_2 2-hydroxylation were not decreased in mature female fish with high levels of circulating E_2. Regulatory factors, such as other hormones present in the mature females, may prevent suppression of E_2 2-hydroxylation by E_2. This is speculative but implies that E_2 is not the only factor regulating the cytochrome P-450 population and monooxygenase activities in female winter flounder.

All of the examined monooxygenase activities, then, were decreased per mg protein in the E_2-treated winter flounder. The response to E_2 resembled the lower specific activities in microsomes from mature female winter flounder than in males. Turnover values for EROD activity were decreased in mature female winter flounder and in the E_2-treated fish relative to males and controls, respectively. E_2 2-hydroxylase turnover values, which were undifferentiated in mature winter flounder or slightly greater in females than in males, were decreased in the experimental fish. Finally, turnover values that were greater in mature female winter flounder than in males---i.e., APDM and testosterone 6β-hydroxylation---were unchanged by E_2 treatment.

Isozyme composition: The specific content of P-450E homolog was decreased in the E_2-treated fish, suggesting that in gonadally mature females endogenous E_2 contributed toward decreasing the specific content of P-450E. The E_2-induced suppression of P-450E was not as great as the sex difference in this parameter, again indicating that a longer period of exposure to E_2, or a secondary factor, is necessary to achieve complete sexual differentiation of P-450E levels. Levels of P-450E homolog relative to spectral cytochrome P-450 were unchanged by E_2 treatment, although they were lower in mature females than in males.

P-450A homolog levels were unchanged by E_2 treatment. This was consistent with the lack of a sex difference in levels of the homolog in adult winter flounder (Table 3-5). In the E_2 treated fish,

P-450A homolog levels were unchanged relative to cytochrome P-450, whereas in adult winter flounder this value was greater in females than in males. Again, this could indicate that factors other than or in addition to E_2 are responsible for sex differences in P-450A and P-450E levels per pmol P-450 in adult winter flounder.

Correlations

There were several significant correlations between levels of microsomal monooxygenase activities and P-450E content when parameters were expressed per mg protein (Table 4-7, top). Specific EROD, APDM, and E_2 2-hydroxylase activities in microsomes from combined control and E_2-treated fish were all correlated with specific P-450E content. In scup microsomes, EROD activity but not APDM or E_2 2-hydroxylase activity was inhibited by MAb 1-12-3 (anti-P-450E) (Klotz et al, 1986; Kloepper-Sams et al, 1987). Therefore positive correlations between the P-450E homolog and APDM and E_2 2-hydroxylase activities per mg protein probably do not indicate causal relationships. The positive relationship indicates only that in microsomes with high levels of catalytic activity per unit protein, there were also high levels of P-450E equivalents.

Correlations were also examined for parameters normalized to microsomal cytochrome P-450 (Table 4-7, bottom). Turnover values for EROD activity were positively correlated with levels of P-450E per nmol spectral P-450, consistent with this homolog being a major EROD catalyst in winter flounder (Stegeman et al, 1987). The regression curves are shown in Figure 4-2. P-450E levels were not correlated with any of the other monooxygenase activities examined.

The relationships between P-450E and EROD activity fit in well with predictions, and therefore correlations between P-450A homolog content and catalytic activities were examined. The specific content of the P-450A homolog was not correlated with any of the monooxygenase activities in the experimental fish. In mature winter flounder, P-450A was only correlated with testosterone 6β-hydroxylase activity. As in adult winter flounder, P-450A homolog levels per pmol spectral P-450 were negatively correlated with P-450E levels and EROD turnover values. Of the remaining activities, P-450A per pmol P-450 was positively correlated only with turnover values for testosterone 6β-hydroxylation. This added support to the hypothesis that the

TABLE 4-7: CORRELATION COEFFICIENTS BETWEEN MICROSOMAL P-450 ISOZYME CONTENT AND MONOOXYGENASE ACTIVITIES IN CONTROL AND E_2-TREATED WINTER FLOUNDER

	EROD/mg	APDM/mg	T 6β-OH/mg	E_2 2-OH/mg	P-450E/mg
APDM/mg[d]	0.852[c]				
T 6β-OH/mg	0.509[a]	0.406			
E_2 2-OH/mg	0.962[c]	0.809[c]	0.558[a]		
P-450E/mg[e]	0.873[c]	0.778[c]	0.374	0.826[c]	
P-450A/mg	0.287	0.324	0.223	0.318	0.287

	EROD TO	APDM TO	T 6β-OH TO	E_2 2-OH TO	P-450E/nmol
APDM TO[d]	0.104				
T 6β-OH TO	-0.198	0.235			
E_2 2-OH TO	0.661[b]	0.232	0.274		
P-450E/nmol[e]	0.568[b]	-0.076	-0.230	0.109	
P-450A/nmol	-0.673[b]	0.103	0.480[a]	-0.291	-0.468[a]

[a-c]Significance of correlation coefficients: [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$

[d]Rates of monooxygenase activities expressed per mg microsomal protein or per nmol P-450 (turnover values; TO).

[e]P-450E and P-450A equivalents per mg protein or per nmol P-450.

There were at least 18 degrees of freedom for each comparison.

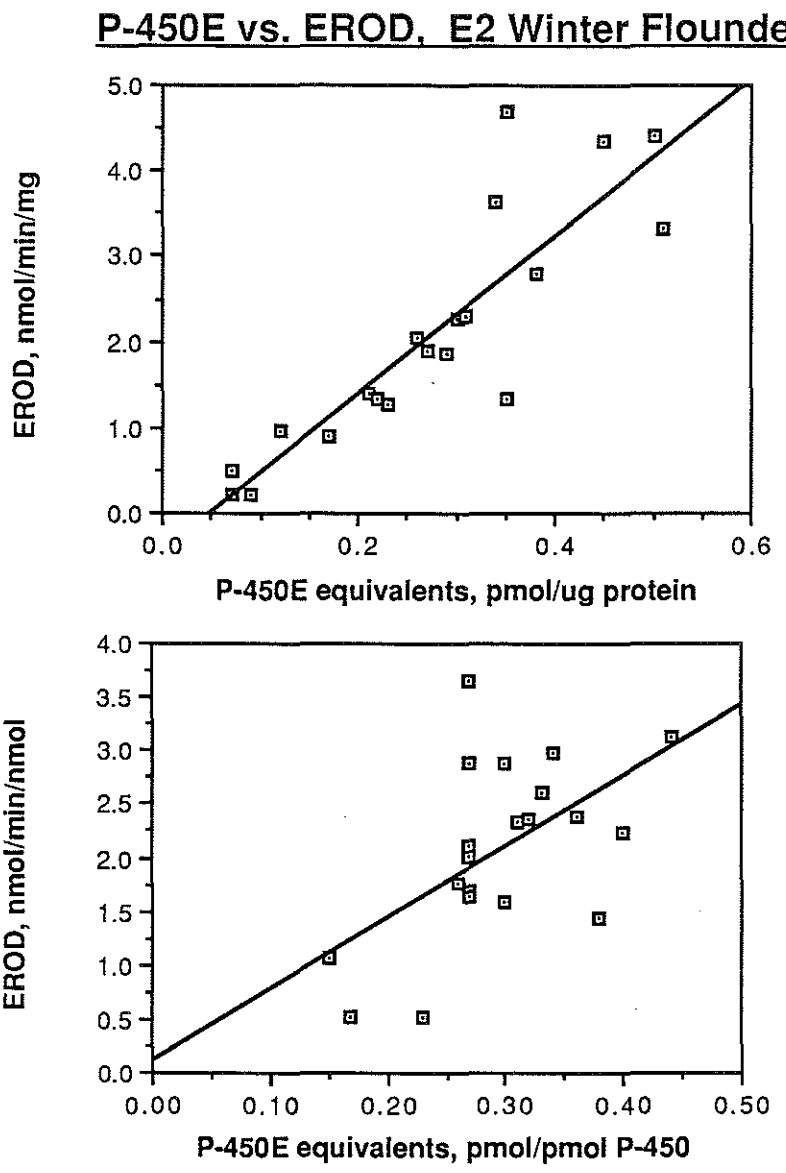


Figure 4-2. Regression between the relative amount of P-450E detected by MAb 1-12-3 and microsomal EROD activity in control and E_2-treated winter flounder. 10 or 25 µg microsomal protein was applied to each lane of gel. Values on the x-axis are the ratio of P-450E equivalents per µg protein or per pmol spectral P-450. N = 20. Above, specific activity: r = 0.873; $P<0.001$. Below, turnover values: r = 0.568; $P<0.01$.

P-450A homolog could be a testosterone 6β-hydroxylase in winter flounder microsomes. The regression curve between P-450A and this activity is shown in Figure 4-3.

There were no significant correlations between turnover values for microsomal APDM and E_2 2-hydroxylase activities in the experimental winter flounder. In adult winter flounder and scup, both activities were positively correlated, normalized either to protein or to cytochrome P-450 (Tables 3-6, 3-7). This observation had suggested coordinate regulation of activities and the possibility of metabolism by the same enzyme. The lack of correlation between APDM and E_2 2-hydroxylation in the experimental winter flounder, though, indicated that in these fish the rates of activity were independent of one another. At least two explanations are possible. Apparently, an E_2 2-hydroxylase catalyst was down-regulated by E_2 treatment. In feral fish, the APDM catalyst may be a separate enzyme closely co-regulated with the E_2 2-hydroxylase catalyst, but not subject to suppression by E_2 treatment. Alternatively, the second enzyme may catalyze both activities, but suppression of the first enzyme by E_2 treatment reduced microsomal E_2 2-hydroxylation to the extent that it was no longer correlated with APDM activity. A preliminary study indicated that E_2 2-hydroxylation by microsomes from feral fish was inhibited by aminopyrine, suggesting that an APDM/E_2 2-hydroxylase catalyst may indeed be present.

Interestingly, there was a positive correlation between turnover values for microsomal EROD activity and E_2 2-hydroxylase activity in the winter flounder used in the experiment (Table 4-7). In scup microsomes, anti-P-450E did not affect E_2 2-hydroxylase activity, and turnover values decreased as the EROD turnover values increased in BNF-treated scup (Snowberger and Stegeman, 1987). This indicated that P-450E was not an E_2 2-hydroxylase and that as P-450E levels increased within the microsomal pool, turnover values for E_2 2-hydroxylation were decreased. Therefore, observing a positive relationship between the E_2 2-hydroxylation and EROD turnover values in winter flounder microsomes seemed counterintuitive. Previously, a positive correlation was observed between these turnover values in microsomes from BNF-treated gonadally regressed winter flounder ($r = 0.72$, $N = 14$, $P<0.01$; Gray, unpub. obs.). The E_2 2-hydroxylase

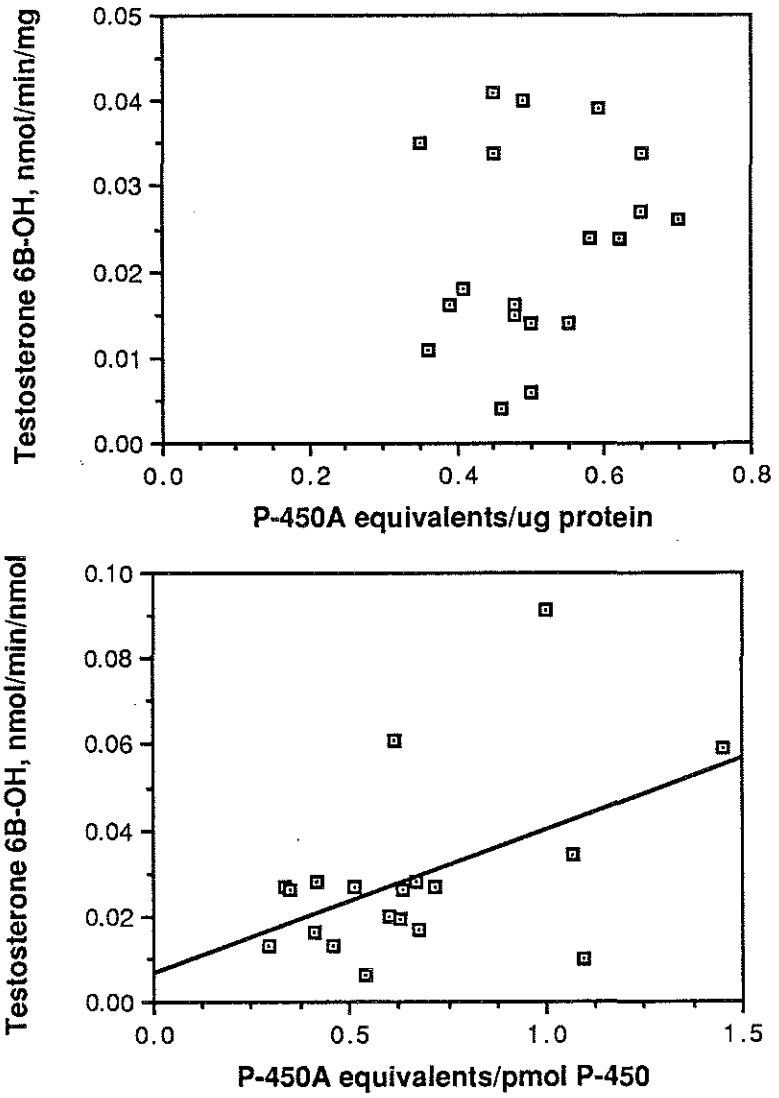


Figure 4-3. Regression between the relative amount of P-450A equivalents detected by anti-P-450A and microsomal testosterone 6β-hydroxylase activity in control and E_2-treated winter flounder. 40 µg microsomal protein was applied to each lane of gel. Values on the x-axis are the ratio of P-450A equivalents per µg protein or per pmol spectral P-450. N = 19. Above, specific activity: r = 0.223; not significant. Below, turnover values: r = 0.480; $P<0.05$.

values were unusually low compared to other winter flounder microsomes, though, rendering the biological significance of the correlation questionable. Further analysis would be necessary to determine if there is a biologically meaningful association between the two activities.

Conclusions

E_2 Imparts Some Sex Differences in Monooxygenase Activities

In reports of sex differences in microsomal metabolism by gonadally mature teleosts, activities per mg protein were in general higher in males than in females (Hansson and Gustafsson, 1981a; Lindstrom-Seppa et al, 1981; Koivusaari et al, 1981). Monooxygenase activities per mg protein were suppressed in two trout species by E_2 treatment (Hansson and Gustafsson, 1981b; Stegeman et al, 1982). In the experiment here, the role of E_2 in effecting sex differences in winter flounder was strongly suggested by the consistently observed decreases in specific monooxygenase activities following E_2 treatment. These decreases were also recorded for cytochromes P-450 and b_5, and for NADPH cytochrome c reductase activity. Vitellogenesis in both experimental fish and mature females involves synthesis of large quantities of hepatic protein, which could interfere with monooxygenase activities in general. Furthermore, ribosomes in the microsomes, believed present due to the known proliferation of the rough endoplasmic reticulum (van Bohemen et al, 1981; Selman and Wallace, 1983), may have somewhat diluted signals normalized to microsomal protein without specifically interfering with the actual monooxygenase reactions.

Sex differences in monooxygenase activities may also stem from E_2 specifically and selectively altering the levels of some of the catalysts. The ratios of specific activities by mature male and female winter flounder were not the same for every microsomal characteristic examined, suggesting that a generalized suppression of activities normalized to protein was not the sole source of the sex differences. Sex differences were observed in the turnover values for EROD, APDM, and testosterone 6β-hydroxylase activities, indicating altered efficiencies of the P-450 pool in mature winter flounder. E_2 treatment decreased the turnover values for EROD activity, reproducing the sex difference in adults. The magnitude of the effect

of E_2 was not as great as the sex difference in mature teleosts, but this may be related to the relatively short (one week) exposure to the hormone. Nevertheless, the suppression of EROD turnover values by E_2 suggested that E_2 in mature females may be responsible for the observed sex difference in EROD activity.

E_2 treatment suppressed P-450E levels per mg protein and EROD per nmol P-450, but P-450E levels relative to cytochrome P-450 were unchanged. Previously, P-450E levels had correlated well with EROD activity in winter flounder (Stegeman et al, 1987). However, in these fish r^2, an estimate of the proportion of variation in EROD due to variation in P-450E, was only 0.32 for activities normalized to spectral cytochrome P-450. The low r^2 value might indicate activity by a second EROD catalyst, although no such catalyst is known to exist. To date, no cytochrome P-450 isozymes have been purified from winter flounder, and specific catalysts of monooxygenase activities in this species have yet to be assigned.

Participation of Factors Other than E_2

E_2 treatment did not reproduce the sex differences observed in the turnover values for microsomeal APDM, E_2 2-hydroxylation, and testosterone 6β-hydroxylation in mature winter flounder. E_2 treatment did not affect turnover values for APDM and testosterone 6β-hydroxylation, yet turnover values for E_2 2-hydroxylation were decreased. Furthermore, levels of P-450A and P-450E homologs did not change toward levels in adult females. This would suggest that in the adult females, E_2 is not solely responsible for regulating monooxygenases, and that other regulatory factors may contribute. There are female-specific hormones other than E_2 in the mature adult. Plasma testosterone levels were elevated in maturing female winter flounder (Wingfield and Grimm, 1977), and could assist in inducing female patterns of metabolism. Hansson (1982) determined with rainbow trout that treatment with testosterone, 11-ketotestosterone, or the combination of E_2 and testosterone modulated microsomal androstenedione metabolism. In winter flounder, then, E_2 and testosterone may act together to produce the female-like pattern of substrate metabolism in the adult.

Another possible effector of sex differences in adult winter flounder is the female specific steroid hormone 17α,20β-dihydroxy-

progesterone (17,20β-P). This appears to be the oocyte-maturation inducing steroid in many teleosts including winter flounder (Scott and Canario, 1987). Plasma 17,20β-P levels were elevated in female winter flounder during spawning, although levels did not appear to correspond with oocyte development (Campbell et al, 1976). The role of this hormone is not completely understood, and it may have physiological roles other than stimulation of oocyte maturation. It remains to be tested if 17,20β-P, alone or in concert with E_2, may regulate monooxygenases in adult female winter flounder.

The influence of pituitary factors on monooxygenation in teleosts is largely unknown. As discussed in the introduction, sex differences in rat cytochrome P-450 are regulated by sex steroids through pituitary hormones. Pituitary extracts from adult salmon partially feminized the specific content of cytochromes P-450 and b_5 by decreasing these components when injected into gonadectomized brook trout (Pajor, 1982). Partially purified salmon GtH did not affect cytochromes P-450 and b_5 in gonadectomized trout, indicating that this was not the pituitary feminizing factor (Pajor, 1982). In hypophysectomized male killifish the specific contents of these two microsomal components were suppressed by pituitary extracts while treatment with cloned salmon growth hormone was without effect (Stegeman, Singh et al, unpub. obs.). These studies suggest that pituitary factors may influence the levels of monooxygenases in teleosts, but do not clearly indicate which factor(s) are involved and if secondary tissues, such as the gonads, also participate.

CHAPTER 5: SUMMARY AND CONCLUSIONS

Estradiol's Role in Sexual Differentiation of Monooxygenases

Sex differences in the cytochrome P-450 monooxygenase system in rats have been attributed to regulation of monooxygenases by endogenous sex steroids and pituitary hormones (Gustafsson et al, 1980). Sex differences in teleosts have also been reported, but little is known of the regulation of teleost cytochromes P-450 by steroid hormones. Monooxygenase activities in two freshwater species of trout were affected by treatment with sex steroids including E_2 (Hansson, 1982; Stegeman et al, 1982). In the first of the studies presented here, microsomal metabolism of E_2 and the regulation of E_2 2-hydroxylase activity by gender and exogenous factors were investigated in two marine teleosts, scup and winter flounder. Following that, patterns of sexual differentiation in several other microsomal monooxygenase activities and specific P-450 isozymes were characterized in both species. Finally, regulation of monooxygenases by E_2 in adult female winter flounder was investigated by treating gonadally immature fish with E_2.

E_2 metabolites formed in vitro by microsomes from scup and winter flounder included at least seven products detected by thin-layer chromatography and high-performance liquid chromatography. The least polar metabolite was shown to be estrone by chromatographic and mass spectrometric identity with authentic estrone. Chromatographic analyses coupled with dual-label experiments also indicated formation of the catecholestrogen 2-OH-E_2, which was the most prominent metabolite determined by TLC. 2-OH-E_2 is a biologically active metabolite of E_2 in mammalian species, and has demonstrated both estrogenic and antiestrogenic properties. Analysis of teleost microsomal E_2 2-hydroxylase activity by measuring specific release of ^3H_2O from [2-^{3}H]E_2 indicated that it was mediated by cytochrome P-450. E_2 2-hydroxylase activity per nmol cytochrome P-450 was reduced in scup treated with β-naphthoflavone, which induced the hydrocarbon hydroxylase cytochrome P-450E. Activity per mg microsomal protein was unchanged by BNF-treatment. Studies employing reconstituted P-450E and microsomes preincubated with polyclonal antibodies against P-450E confirmed that this isozyme does not catalyze E_2 2-hydroxylase activity in scup microsomes

(Snowberger and Stegeman, 1987). Reconstituted cytochrome P-450A, a testosterone 6β-hydroxylase, displayed limited E_2 2-hydroxylase activity (Klotz et al, 1986).

Antibodies were generated against scup P-450A and a second scup isozyme, P-450B. Both isozymes were electroeluted in denatured form from SDS-PAGE gels and used to immunize rabbits. The resulting polyclonal antibodies recognized their respective scup P-450s, as determined by immunoblot. Proteins in microsomes from winter flounder, killifish, and rainbow trout were also recognized by these antibodies. The antibodies did not inhibit catalytic activity in scup microsomes, and were therefore employed exclusively in immunoblots. Because reconstituted P-450A was an apparent testosterone 6β-hydroxylase, a sexually differentiated activity in winter flounder microsomes (Stegeman and Woodin, 1984), studies focused on this isozyme. Correlations were calculated between levels of microsomal activity and P-450A levels, as determined by immunoblot, to suggest which activities might be conducted by this isozyme.

In scup, levels of cytochrome P-450 per mg protein were not sexually differentiated in prespawning fish collected in May, but were higher in male scup collected in June than in females collected at the same time. Specific EROD activity was low in both sexes of scup collected in May; in June, EROD activity was also low in female scup, but were elevated in males. Turnover values for EROD activity were higher in female than male May scup, and higher in male than female June scup. Specific APDM and testosterone 6β-hydroxylase activities were not sexually differentiated, but specific E_2 2-hydroxylase activities were lower in female scup than in males. Turnover values for APDM, E_2 2-hydroxylase, and testosterone 6β-hydroxylase activities were not sexually differentiated. The specific content of P-450E was low in scup collected in May and in female scup collected in June, while in male June scup P-450E per mg protein was increased. Within the June scup, the sex difference in P-450E content reflected the sex difference in specific EROD activity. P-450A was not sexually differentiated per mg protein or per nmol P-450 in mature scup. Correlation analyses indicated that P-450A could catalyze microsomal E_2 2-hydroxylase activity, but might not be the primary testosterone 6β-hydroxylase catalyst. An additional observation was that

microsomal APDM and E_2 2-hydroxylase activities were positively correlated, suggesting that they were co-regulated.

In winter flounder, levels of microsomal cytochrome P-450 were substantially lower in gonadally mature females than in males. Both the specific activity and turnover values for EROD were depressed in females. Specific APDM activity was decreased in females while testosterone 6β-hydroxylase activity was not sexually differentiated; turnover values of both were greater in females than in males (Stegeman and Woodin, 1984; Chapter 3). Specific E_2 2-hydroxylase activity was decreased in females, while turnover values for E_2 2-hydroxylation in females were slightly greater than or equal to those in males. Levels of the P-450E analog per mg protein or per nmol P-450 were decreased in female winter flounder. EROD activity and P-450E levels were positively correlated with each other in winter flounder, reflecting the sex difference in EROD activity and the strong evidence that the P-450E homolog is the major EROD catalyst in this species. P-450A levels per mg protein were not sexually differentiated, but P-450A levels per nmol P-450 were greater in females than in males. Correlation analyses indicated that testosterone 6β-hydroxylase activity might be conducted by the P-450A homolog, and that the P-450A homolog probably did not catalyze APDM and E_2 2-hydroxylase activities. These latter two activities were positively correlated in winter flounder microsomes, as in scup microsomes, suggesting catalysis by co-regulated enzymes or even by the same enzyme.

Previous studies with trout had shown that E_2 treatment regulated cytochrome P-450 and P-450-mediated activities (Hansson, 1982; Stegeman et al, 1982). Because sex differences in microsomal monooxygenases were so strongly pronounced in mature winter flounder, this species was selected to test of E_2 might regulate monooxygenase activity in female adults. Two injections of E_2 within one week reduced the specific content of microsomal cytochrome P-450, and specific EROD, APDM, testosterone 6β-hydroxylase, and E_2 2-hydroxylase activities. This resembled the patterns of suppressed specific activities in adult female winter flounder. The specific content of the P-450E analog was decreased, but P-450E levels relative to total microsomal cytochrome P-450 were unaltered. P-450A homolog

levels were unchanged, whether normalized to protein or to cytochrome P-450. Turnover values for EROD, previously shown to be lower in adult females than in males, were decreased by E_2 treatment. E_2 2-hydroxylase turnover values were also decreased by E_2, yet were not sexually differentiated in adults. APDM and testosterone 6β-hydroxylase turnover values were unchanged by E_2 treatment, although in adults both were greater in females than in males.

Hence, E_2 treatment of winter flounder partially induced some characteristics of adult females, such as suppression of EROD turnover values and suppression of several microsomal monooxygenases normalized to microsomal protein. However, E_2 did not induce female-like patterns of APDM and testosterone 6β-hydroxylase activities, and turnover values for E_2 2-hydroxylation, which were not decreased in adult females, were suppressed by E_2 treatment. This suggested that in adult females, decreases in specific activity can result from E_2-directed general suppression of monooxygenases, but sex differences in turnover values, indicating regulation of particular P-450 isozymes within the microsomal P-450 pool, is directed by additional factors. These factors could include testosterone or 17,20β-P, which are both present in plasma from prespawning female winter flounder.

In experimental fish, as in adults, turnover values for microsomal testosterone 6β-hydroxylase activity were correlated with P-450A content per pmol P-450, supporting the idea that P-450A is a microsomal 6β-hydroxylase. There was no correlation between microsomal APDM and E_2 2-hydroxylase activities. This contrasted with their being positively correlated in untreated adult winter flounder, which had suggested metabolism by the same enzyme or by co-regulated enzymes. Possibly, APDM and E_2 2-hydroxylase activities were co-regulated in the adult fish, but under experimental conditions, E_2 treatment only affected the levels of the E_2 2-hydroxylase.

Regulation of P-450 by E_2, then, appears to contribute toward sexual differentiation of monooxygenase activity in mature winter flounder, but participation of other factors is indicated. Turnover values for microsomal EROD activity decreased following E_2 treatment, suggesting that in adult females E_2 could contribute

toward depressing turnover values for EROD. P-450E per nmol cytochrome P-450 was not altered by E_2 treatment although levels were decreased in mature females. It is unclear if suppression of EROD turnover values in immature winter flounder following E_2 treatment was effected by the same mechanism suppressing EROD activity in adult females. The lack of an effect of E_2 on other turnover values for microsomal activity suggested that this treatment regime was insufficient to induce patterns of metabolism present in the adult female. The relatively short exposure period to E_2 (one week) may have been insufficent to induce female-like patterns of metabolism in the gonadally immature winter flounder. In addition, other hormones of the adult female may participate in regulating cytochrome P-450.

Speculation

The reason for sex differences in teleost monooxygenases is unclear. To date, there has not been a satisfactory reason proposed for sex differences in monooxygenases in rats, and other mammalian species have certainly thrived without obvious sexually differentiated levels of cytochrome P-450 mediated activities. Pre-spawning female teleosts synthesize large quantities of protein in the liver, and it may be advantageous for them to suppress the levels of other hepatic proteins deemed temporarily unnecessary. However, suppression of P-450E, an isozyme involved in removing foreign compounds, does not appear to be desirable. Perhaps it is advantageous to suppress metabolism of an endogenous P-450E substrate, but none has been identified. In addition to the sex difference in P-450E levels, P-450A was differentiated, with levels per nmol P-450 elevated in pre-spawning female winter flounder. Too little is known of the winter flounder P-450A homolog to speculate on its role, but this isozyme may have important catalytic function necessitating its presence during reproduction.

It is interesting to consider how sex differences in monooxygenase activity may affect teleosts living in a polluted environment, such as the coast of Massachusetts. Decreased levels of the P-450E homolog in female teleosts could impair their ability to rid themselves of xenobiotics. Winter flounder enter coastal waters by October, and suppression of EROD activity in females was observed by November and maintained for at least two months. This represents a

fairly long period during which females were exposed to elevated levels of xenobiotics with an apparently reduced capacity to metabolize and excrete them. However, in female teleosts transfer of lipophilic xenobiotics to gametes is an effective route of elimination which may somewhat reduce viability of the next generation, but allows mature females to discharge harmful foreign compounds (Binder et al, 1984; von Westernhagen et al, 1981). P-450E is proposed to be linked with initiation of carcinogenesis in fish by inadvertant activation of xenobiotics to reactive intermediates (Stegeman and Kloepper-Sams, 1987). Suppression of P-450E may protect females from this particular hazard. However, because P-450E levels were reduced for only part of the female winter flounder's lifecycle, it seems unlikely that long-term sex differences in winter flounder health would result directly from temporary suppression of P-450E.

APPENDIX: RESULTS OF PRELIMINARY IMMUNOBLOTS AGAINST ANTI-P-450B

As described in chapter 3, polyclonal antibodies against scup P-450B were obtained during the course of isolating P-450A and generating antibodies against that isozyme. Levels of the isozyme and its homolog in scup and winter flounder microsomes, respectively, were estimated with immunoblots against anti-P-450B. Microsomal proteins were separated on SDS-PAGE and blotted onto nitrocellulose as described in chapter 3. P-450B was included in each gel as a positive control. Nitrocellulose sheets were then incubated with anti-P-450B, 20 µg/ml, and developed as described. Intensity of staining was quantitated by densitometric scanning. Scanning results were not transformed to P-450B equivalents, but presented as absorbance units. Comparisons were made between microsomes separated on the same gel and blotted onto the same nitrocellulose. The anti-P-450B reactive protein in winter flounder microsomes is here referred to as the P-450B homolog.

The levels of P-450B per mg microsomal protein did not appear to be differentiated by BNF-treatment in microsomes from either scup or winter flounder (Table A-1). Levels of P-450B per nmole P-450 were also not affected by BNF treatment. In contrast, immunoblots confirmed that P-450E was induced in the BNF-treated fish (J. Gooch and A. Elskus, pers. comm.; Stegeman et al, 1987). As a first approximation, then, microsomal P-450B and its winter flounder homolog do not appear to be induced or suppressed by the MC-type inducer BNF.

In winter flounder, the specific content of the P-450B homolog was greater in microsomes from mature males than from mature females. This is like the suppression of P-450E and several monooxygenase activities per mg microsmal protein. P-450B levels relative to cytochrome P-450 were not sexually differentiated. P-450B levels were measured in only a few samples, but it appears that the P-450B homolog per mg protein may be suppressed in female winter flounder while levels per nmole cytochrome P-450 were not sexually differentiated. This is different from the regulation of the P-450E and P-450A homologs, both of which were sexually differentiated per nmole P-450 in winter flounder. Microsomes from gonadally mature scup were not assayed for P-450B content.

TABLE A-1: MICROSOMAL P-450B IN SCUP AND WINTER FLOUNDER

Species	P-450B, unit[b]/mg	P-450B, unit/nmol P-450
Scup		
Control (4)[c]	25 ± 34	106 ± 114
BNF-treated (4)	36 ± 24	109 ± 49
Winter flounder		
Control (7)[d]	27 ± 21	69 ± 63
BNF-treated (7)	61 ± 26	78 ± 29
Mature males (4)[e]	65 ± 35[a]	49 ± 19
Mature females(4)	4 ± 3	28 ± 21

[a]Significantly different from mature females, $P<0.05$. Mean for males included one relatively high data point; excluding this one, the mean for males was still significantly greater than for females.
[b]Results of densitometric scanning are presented as arbitrary units per mg protein or per nmol P-450.
[c]Microsomes provided by J. Gooch and A. Elskus. Scup were injected on day 0 with corn oil or 20 mg/kg BNF and sacrificed on day 5.
[d]Winter flounder were injected with BNF or corn oil vehicle as described in chapter 3. Samples were identical to those utilized in Stegeman et al, 1987.
[e]Microsomes from male and female winter flounder are a subset from those analyzed for P-450A and P-450E, Table 3-5.

From these preliminary observations, then, the regulation of P-450B and its winter flounder homolog appears to be unlike that of either P-450E and P-450A. Scup P-450B is a tentative testosterone 15α-hydroxylase (Klotz et al, 1986), but it is not known what other activities are catalyzed by the isozyme. Further investigations are required to determine the regulation and function of P-450B and its winter flounder homolog.

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This book is set in 12-point Monotone Bimbo, with chapter headings in Basketball overextended. Both faces were designed by the great Adolf Pflupfl and are characterized by noble, full-bodied proportions with complex, slightly fruity serifs. It was printed by upset lithophagy on 70-lb Tropicana Ivory Mislaid Cowabunga Slipshod Overcoat. The ink came out of a can.

Tom Weller
Science Made Stupid